IN VIVO ANTIMALARIAL ACTIVITY, TOXICITY AND PHYTOCHEMICAL SCREENING OF AQUEOUS AND ORGANIC EXTRACTS OF SELECTED ANTIMALARIAL PLANTS IN MSAMBWENI DISTRICT, KENYA.

By

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DECLARATION

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DEDICATION

This thesis is dedicated to my family members and friends who provided me with moral and financial support throughout my studies.

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LIST OF ABBREVIATIONS

ANOVA:	Analysis of Variance
DMSO:	Dimethylsulphoxide
DV:	Dependent Variable
DW:	Distilled Water
CQ:	Chloroquine
$CHCl_3$:	Chloroform
CTMDR:	Center for Traditional Medicine and Drug Research
H_2SO_4:	Sulphuric acid
IV:	Independent Variable
KEMRI:	Kenya Medical Research Institute
LD_{50}:	Lethal Dose 50
MeOH:	Methanol
MF:	Magnification Field
PBS:	Phosphate Buffered Saline
SPSS:	Statistical Package for the Social Sciences
TLC:	Thin Layer Chromatography
UV Light:	Ultra Violet Light

ABSTRACT

Malaria continues to kill over a million people each year, with more than 90% of these cases found in sub-Saharan Africa. In many populations affected by malaria, conventional drugs are often unaffordable or inaccessible, and increasing drug resistance by the malaria parasite, *P. falciparum*, is of significant concern. The current study involved determination of antimalarial activity and toxicity of selected plants from Msambweni district, Coast province; Kenya.

Aqueous and organic [Chloroform: Methanol (1:1)] extracts from each plant were prepared and used to determine *in vivo* anti-malarial activity and toxicity. They were also screened for their phytochemical constituents. To evaluate antimalarial activity, Swiss albino mice (*Mus musculus* L.) were infected with *Plasmodium berghei* parasites through intraperitoneal route. Crude extracts were administered orally everyday at the same hour for four days at a dosage of 100 mg/kg from Day 0 to Day 3. Thin blood smears were prepared on Day 4 from each mouse and stained with 10% Giemsa in phosphate buffer, pH 7.2 and examined microscopically for assessment of parasitaemia. The mean parasitaemia in each group of mice on Day 4 was used to calculate the percentage chemosuppression for each extract.

Toxicity of the crude extracts was evaluated using Brine shrimp larvae (*Artemia salina* L. *nauplii*). ANOVA was used to analyze the means of the parasite growth inhibitions while LD_{50} was estimated using Finney's probit analysis program. The screened plants exhibited varying degrees of chemosuppression. Aqueous extracts of *Adansonia digitata* L. (Bombacaceae), *Zanthoxylum chalybeum* Engl. (Rutaceae), *Launea cornuta* (Hocht. ex Oliv. & Hern) C.Jeffrey (Compositae) and *Canthium glaucum* Hiern. (Rubiaceae) had 60.47%, 44.93%, 38.13% and 31.98% parasite growth inhibition respectively. $CHCl_3$: MeOH extracts of *C.glaucum* and

A.digitata showed parasite growth inhibition of 43.76% and 32.90% respectively. Chloroquine (positive control) had 87.23% parasite growth inhibition.

Crude extracts of *A.digitata* and *C.glaucum* had LD_{50}>1000 µg/ml and were considered to be non toxic to Brine shrimp larvae unlike crude extracts of *Z.chalybeum* and *L.cornuta* which had LD_{50}<500 ug/ml. Phytochemical screening of the crude extracts showed that alkaloids, flavonoids, sesquiterpene lactones and saponins were present in *A.digitata*, *C.glaucum*, *L.cornuta* and *Z.chalybeum*.

This study reports for the first time the *in vivo* antimalarial activity of the crude root (aqueous and organic) extracts of *C.glaucum* and crude leaf (aqueous and organic) extracts of *L.cornuta*. In addition the toxicity of aqueous and organic root extracts of *C.glaucum* is also being reported for the first time. From the study *A.digitata* and *C.glaucum* have been shown to possess promising antimalarial activity and were not toxic to the Brine shrimp larvae. These results indicate that there is potential for isolation of new scaffolds against *Plasmodium falciparum* from the aqueous extracts of the two plants.

Key words

Plasmodium berghei, Adansonia digitata, Canthium glaucum, Launaea cornuta, Zanthoxylum chalybeum, Mus musculus (Swiss albino mouse), *Artemia salina* (Brine shrimp), Toxicity, *In vivo* anti-malarial activity and Phytochemical screening

1. CHAPTER ONE: INTRODUCTION

1.1. General Introduction

Malaria continues to kill over a million people each year, with more than 90% of these cases found in sub-Saharan Africa (Nguta *et al.*, 2010). In many populations affected by malaria, conventional drugs are often unaffordable or inaccessible, and increasing drug resistance by the malaria parasite, *P. falciparum*, is of significant concern (Greenwood and Mutabingwa, 2002). As an alternative, medicinal plants are often used to treat diseases such as malaria and development of new drugs is very important to cut down the malaria scourge (Ajaiyeoba *et al.*, 1999). Vector resistance to drugs and mutating parasites has made it hard to control the spread of the disease (Hilou *et al.*, 2006). In addition most antimalarial drugs are not affordable to most people especially those living in the third world countries as a result new cheap drugs are needed.

Most drugs for malaria are expensive and not accessible to many locals who live away from health centers (WHO, 2004). The ever-rising cost of modern medicine and its technical inadequacies has prompted many people particularly in developing countries to result to traditional medicine to treat a variety of ailments they succumb to (Kokwaro, 2009).Traditional remedies are employed by many communities to cure malaria and other infectious diseases (Murray, 1995). Since time immemorial, man has depended on plants as the primary source of medicine to cure a variety of diseases. Various plants have been used by man in folk medicine to treat malaria (Kokwaro , 2009)

In the past, drugs have been developed from plants. For example major drugs such as quinine and artemisinin have been derived from traditional medicine (Nguta *et al.*, 2010). Artemisinin was derived from a leafy portion of a Chinese traditional herb known as *Artemisia annua* L.

(Compositae) (Akira *et al.*, 2001). Plants provide rich source of drugs for treatment of malaria as has been argued by Julie *et al.*, (2007). From ethnomedicine data, it is documented that many plants are used in control and treatment of malaria however very few of these plants have been analyzed chemically (Garavito *et al.*, 2006). As a result there is great need to analyze these plants which form the basis of ethnomedicine and are used to treat malaria to ascertain whether they have effects on the malaria parasites.

On the other hand, plants have toxic effects on livestock and humans, such toxicity may be high leading to death of an animal or humans consuming the plant. In traditional medicine, one is likely to overdose the patient due to imprecise nature of diagnosis. This is not only unique to traditional medicine but can also occur in modern medicine. Toxicity is attributed to certain acting principles found in drugs; these chemical substances interact with living systems and affect normal processes. All chemicals can cause harm to organisms at some level of exposure. Toxicity tests are important so as to determine the lethality of drugs and to determine the harmless concentration of drugs for consumption (Hood, 2009). This ensures and enhances human health, animal health and protection of the environment.

Plants produce array of secondary metabolites which exhibit a broad spectrum of biological activity. These include: alkaloids, sesquiterpene lactones, flavonoids, steroids, athraquinones and essential oils besides others (Mazid *et al.*, 2011). Biological activities of plants extracts include antifungal, anti-malarial, antibacterial, anti-fertility, cytotoxic, larvicidal and insecticidal activities among others (Mazid *et al.*, 2011). Biological activity of plants is attributed to the class and concentration of phytochemical constituents in the plants which makes some plant extracts exhibit a variety of activities (Wang *et al.*, 2010). Phytochemical screening of plant extracts

especially those which have been used in traditional medicine is therefore essential so as to identify phytochemical constituents in the plants that are responsible for a given bioactivity.

Majority of malaria drugs in current use have developed resistance against *Plasmodium* parasites. For instance, chloroquine resistance strains of *P.falciparum* have been reported (David *et al.*, 2004). Other drugs such as amodiaquine and lapdap suffer cross-resistance with chloroquine and fansidar (David *et al.*, 2004). Nevertheless various strains of *Plasmodium* parasites have emanated which are tolerant to Artemisinin based combination therapies (ACTs) which are considered as the best drugs for treatment of malaria (Tolu *et al.*, 2007). Geographical resistance to malaria drugs has also been reported which entails resistance to anti-malarial drugs in various geographical areas (Shretta *et al.,* 2000). An ideal drug for treatment of malaria should be able to cure within a short period of time, safe, it should be less toxic, suitable for expectant mothers and small children who are most vulnerable to malaria, it should be efficacious against drug resistant strains and should be affordable by most people (David *et al.*, 2004).

Since *Plasmodium* parasites have developed resistance to most of the current drugs for malaria, new anti-malarial drugs are needed urgently to counteract the disease. Regions which are endemic to malaria are faced with serious situation because the only available malaria drugs are losing therapeutic value. Plant biodiversity provides one untapped source for new anti-malarial drugs. This is supported by ethnomedicine where various plants have been used to treat malaria. As a result numerous malaria development and discovery projects are underway. In the process of evaluation of anti-malarial properties of plants and in drug discovery; drug efficacy, pharmacology and toxicity are important parameters which need to be considered (David *et al.*, 2004).

2. CHAPTER TWO: LITRATURE REVIEW

2.1. Malaria prevalence and its socio-economic impacts

Malaria is one of the most important human infectious diseases in the world. It kills about 1 to 3 million people and cause disease in 300-500 million people annually (UNICEF, 2007). Malaria is caused by protozoan parasites of the genus *Plasmodium*. These malaria causing parasites are host-specific. The four *Plasmodium* parasites infecting humans include *P. falciparum*, *P.vivax*, *P.ovale* and *P.malariae*. Of these four parasites, *P. falciparum* is the most severe in terms of infection rates and the pathology it causes because it produces more merozoites in blood than any other plasmodium species (Robert *et al.*, 2005; Carico *et al.*, 2004). Other *Plasmodium* parasites include *P.knowlesi* which causes malaria in monkeys; *Plasmodium berghei, Plasmodium yoelii*, *P.vinckei* and *P.chabaudi* are malaria causing parasites in rats (Sinden, 1978).

Malaria is frequently referred to as disease of the poor, because it is concentrated in world's poorest countries. Sub-Saharan Africa is the region which is hardest hit by malaria with most countries in the region being highly endemic for malaria transmission (UNICEF, 2007). Low land areas which experience high temperatures have high malaria prevalence unlike highland areas and deserts fringes. This is because temperature favors the life cycle of the malaria parasite vector. Children, pregnant women and people living with HIV are at high of contacting malaria. Malaria mostly affects low income earners who live in poorly constructed houses (Worral *et al.*, 2003; UNICEF, 2007). Most of these people have low levels of education and live in unhygienic environment. Malaria incidents are high in rural areas compared to urban areas due to socio-economic factors such as differences in education and income (Cox *et al.*, 1999).

The burden of malaria is high among world's poorest countries unlike in rich countries which only account for 0.2% of the global malaria deaths. In terms of Disability Adjusted Life Years (DALYS), the burden is high in poor countries of the world (Worral *et al.*, 2003). Malaria is expensive to treat and an estimated loss of $12 billion per year in GDP is encountered in treatment of malaria in most countries of Sub-Saharan Africa. Malaria increases government health spending and reduces the income of the common man because it is expensive to treat (Worral *et al.*, 2003). Malaria keeps poor people poor and this is a drawback to economic development. Malaria is a serious and debilitating disease with negative side effects to a community and to a given region at large hence infected people ought to be treated soon. Indeed World Health Organization recommends that all children under the age of five with fever must be treated with anti-malarial drugs in areas of high or moderate malaria transmission (UNICEF, 2007)

2.2. Malaria in Kenya

Malaria is the leading cause of morbidity and mortality in Kenya 25 million out of a population of 34 million Kenyans are at risk of malaria. It accounts for 30-50% of all outpatient attendance and 20% of all admissions to health facilities. An estimated 170 million working days are lost to the disease each year (MOH 2010). Malaria is also estimated to cause 20% of all deaths in children under five (MOH 2010). The most vulnerable group to malaria infections are pregnant women and children under 5 years of age.

Zones of seasonal malaria transmission include the arid and semi-arid areas of northern and south-eastern parts of the country which experience short periods of intense malaria transmission during the rainfall seasons (KMSI, 2011). Temperatures are usually high and water pools created during the rainy season provide the malaria vectors breeding sites. Extreme climatic conditions

which cause flooding in these areas lead to epidemic outbreaks with high morbidity rates due to low immune status of the population (DOMC, 2010a)

Malaria transmission in the western highlands of Kenya is seasonal, with considerable year-to-year variation (KMSI, 2011). The epidemic phenomenon is experienced when climatic conditions favor sustainability of minimum temperatures around 18^0 C. This increase in minimum temperatures during the long rains period favors and sustains vector breeding resulting in increased intensity of malaria transmission. The whole population is vulnerable and case fatality rates during an epidemic can be up to ten times greater than what is experienced in regions where malaria occurs regularly (KEMRI FACT SHEET, 2012)

Low risk malaria areas include the central highlands of Kenya including Nairobi (DOMC, 2010b). The temperatures are usually too low to allow completion of the sporogonic cycle of the malaria parasite in the vector (KSMI, 2011). However with increasing temperatures and changes in the hydrological cycle associated with climate change are likely to increase the areas suitable for malaria vector breeding with introduction of malaria transmission in areas it never existed.

The burden of malaria in Kenya is being monitored through several channels including health information data from hospitals and clinics, sentinel sites surveys of communities and health facilities in various districts and national surveys including the Kenya Demographic Health Survey (KSMI, 2011). Evidence from these sources now points towards increased coverage of interventions with a downward trend in disease burden demonstrated by community reported cases of malaria, hospital admissions and deaths due to malaria and childhood deaths from the diseases (KEMRI FACT SHEET, 2012).However, malaria still remains a big threat to human life in Kenya and ways of combating the disease need to be put in place.

2.3. Mouse models and *P.berghei* in drug discovery

Plasmodium berghei is one of the many species of malaria parasites that infect mammals other than humans. *P. berghei* belongs to a group of four *Plasmodium* species that infect murine rodents from Central Africa. These species are *P. vinckei*, *P. chabaudi*, *P. yoelii* and *P. berghei* (Lin *et al.*, 2010; Sinden, 1978). The rodent parasites are not of direct practical concern to man or his domestic animals. The interest of rodent malaria parasites is that they are practical models for the experimental study of mammalian malaria. These parasites have proved to be analogous to the malaria parasites of man and other primates in most essential aspects of structure, physiology and life cycle (Carter and Diggs 1977).Therefore, for investigation of different aspects of human infection one could question whether or not the use of non-human malaria parasites is still appropriate. Rodent parasites and their hosts are diverged from the human parasites and human host and therefore careful comparison and assessment of results from rodent models is essential to assess their relevance for human disease (Sinden, 1978)

In the process of studying human malaria parasites, rodent parasites are recognized as valuable model parasites for the investigation of the developmental biology of malaria parasites, parasite-host interactions, vaccine development and drug testing (Menard *et al.*, 1997). *P. berghei* is an excellent model for research on the developmental biology of malaria parasites, because of the availability of: Technologies for *in vitro* cultivation and large scale production and purification of the different life cycle stages; Knowledge on the genome sequence and organization; Methodologies for genetic modification of the parasite; Well characterized clones and genetically modified mutant lines, including transgenic parasites expressing reporter genes such as Green Fluorescent Protein and Luciferase (Calton *et al.*, 2002; Janse *et al.*, 1993).

The other rodent parasites are invaluable in different areas of malaria research. For example, *P. chabaudi* is recognized as a useful model for investigations of mechanisms of drug resistance and antigenic variation (Peters, 1998). This parasite shows antigenic variation during long lasting, non-lethal, infections in laboratory rodents. In contrast, *P. berghei* infections are usually rapidly lethal to laboratory rodents which hamper studies on the *in vivo* generation and selection of antigenic variants (Philips *et al.*, 1997). Another example is *P. yoelii*, which is extensively used in studies on the biology of liver stage and blood stage antigens and their role in immunity and vaccine development (Mota *et al.*, 2001).

2.4. Relationship between *P.berghei* and *P.falciparum* life cycles

The morphology of the different developmental stages is conserved between mammalian malaria parasites. The life cycles and the different developmental stages of all mammalian malaria parasites are highly comparable. Mammalian malaria parasites share the following characteristics; they are only infectious to Anopheline mosquitoes, haploid sporozoites invade and develop only in liver-cells; after multiplication in the liver the parasites (merozoites) invade and multiply in red blood cells; in the blood a relatively small percentage of parasites develop into gametocytes, the precursor cells of the haploid gametes; fertilization and development of the diploid zygote into ookinetes occur in the midgut of the mosquito; mature ookinetes penetrate the cells of the midgut wall and develop into oocysts on the outside of the midgut (Sinden, 1978; Lin *et al.*, 2000).

The invasive, non-dividing stages such as merozoites, sporozoites, ookinetes and microgametes of the different mammalian malaria parasites are very similar in size and morphology. The similarity of these stages of the different species is clear from investigations on the ultra-structure of these stages (Lin *et al.*, 2000). Increasing evidence coming from molecular studies

also demonstrates the similarity of these stages between the various mammalian *Plasmodium* species (Sinden, 1978). For example, a number of surface proteins demonstrate conservation both in structure and function between rodent and human malaria parasites such as CS, TRAP, P45/48, CTRP, P25, P28, AMA-1, MSP-1 (Lin *et al.*,2000).

The genome organization is conserved between rodent and human malaria parasites. Recent studies demonstrate a high level of conservation of genome organization between rodent and human parasites. The genome of both *P. falciparum* and the four rodent parasites are organized into 14 linear chromosomes, ranging in size from 0.5-3.8 Mb (Rich & Ayala 2003). Metabolic pathways are conserved between mammalian malaria parasites. To our knowledge no gross differences in metabolic pathways between mammalian malaria parasites have been reported (Barnwell & Wertheimer, 1989). The similarity in sensitivity of mammalian malaria parasites to antimalarial drugs and other specific inhibitors emphasizes the similarities in their metabolic processes (Barnwell & Wertheimer, 1989).

Despite the overall similarity between mammalian malaria parasites, many 'small' differences exist that are directly related to interaction of the parasites with their hosts. Differences in the life cycle of *P.berghei* and *P.falciparum* or generally differences between various mammalian malaria parasites life cycles are mainly restricted to the duration of development and size of the different dividing stages (Akawa & Seed, 1980). For example, there are significant differences in developmental time and size of liver schizonts, erythrocytic schizonts and oocysts. These small differences may have a significant impact on host-parasite interactions and may influence important features such as pathology, virulence and immune escape (Waters *et al*, 1991). Generally all mammalian *Plasmodium* parasites induce malaria in a similar manner and studies

of malaria using *P.berghei* and mouse models can be used in the development of drugs against *P.falciparum* in human beings.

2.5. Traditional approach to treatment of malaria

In folk medicine people use decoctions, concoctions and infusions from various plants to treat diseases they encounter. A variety of plants have been used in ethnomedicine by different communities to treat malaria. The plant parts used include leaves, fruits, roots, stem bark as well as whole plant particularly when herbs are used. These drugs are prepared by boiling, soaking in cold water, crushing of plant parts followed by soaking. The extracts can either be drunk alone, mixed with food, soup or even milk to make them palatable. Sometimes a decoction from single plant is administered alone or infusion containing extracts from two or three plants is given to the patient (Kokwaro, 2009). However traditional treatment of malaria and other diseases has several disadvantages such as imprecise nature of diagnosis and over dosage. Nevertheless traditional medicine remains the only source of medicaments to people who are not accessible to modern drugs (Kahunu *et al.*, 2011).

2.6. Treatment of malaria

Clinical manifestations of malaria include: fever, anemia, chills, metabolic acidosis, low birth weight and still birth. Severe symptoms include: cerebral malaria, organ failure, coma and death. Mostly used drugs for treatment of malaria include: chloroquine, artemisinin derivatives and sulphfadoxine pyrimethamine (SP) combination commonly known as fansidar (David *et al.*, 2004). Other malaria drugs are mefloquinine, atavoquone and clindamycin. Artemisinin derived drugs have short half lives and as a result they are combined with other long acting drugs (David *et al.*, 2004; Hombhanje & Huang, 2010). Artemisinin combined therapies (ACTs) are

considered as the best current drugs for treatment of malaria (Gathirwa *et al.*, 2011). Malaria is a major cause of mortality in young children, expectant women and people living with HIV and effective drugs are needed to counteract it. In addition, the mutation rate for malaria parasites is high and currently most malaria parasites have developed resistance to the current malaria medicine (David *et al.*, 2004). As a result, research and development of new malarial drugs is needed.

2.7. Resistance of Malaria parasites to drugs in Kenya

Resistance to antimalarial drugs in Kenya has been described for the four species that infect humans (WHO, 2001). *P. falciparum* has developed resistance nearly to all antimalarials in current use (WHO, 2001). *P.falciparum* in Kenya is moderately multidrug resistance as determined by the various genotype and phenotypic analyses (UNICEF, 2007). Although Malaria parasite mutation is a major cause of drug resistance, Malaria treatment failure which may be due to incorrect dosing, non-compliance with dosing regimen, poor quality drug and misdiagnosis intensifies the intensity of *Plasmodium* parasite resistance due to exposure to suboptimal drug levels (D'Alessandro & Buttiens, 2001). Drug resistance by malaria parasites increases the burden of Malaria for countries like Kenya. Therefore implementation of control measures is needed to reduce the burden of the disease. One way of reducing the burden is coming up with a novel chemotherapy and study of botanicals can be a good way to start.

2.8. Importance of toxicity studies

Many substances we handle daily are toxic. Different organisms respond differently to the same toxin. Lethal Dose 50 is a term which is used to describe acute toxicity. It refers to lethal dose which is acutely lethal to 50% of all the organisms in a given experiment. LD_{50} is expressed in

mg/kg or ppm (parts per million). Toxicity study is a first step necessary for new drug development. Brine shrimp lethality test is a general bioassay which is capable of detecting broad spectrum of bioactivity of crude plant extracts (Chanda & Bravalia, 2011). Plant products are heterogeneous because they contain mixtures of bioactive constituents.

Simple bioassays for determination of bioactivities of crude plant extracts are important for the purpose of standardization or quality control of plant botanicals. Due to this Brine shrimp lethality test is a "top bench" procedure useful in natural product chemistry (Jerry & Lingling, 1998). Using Brine shrimp in toxicity studies is economical, easy, uses small amount of test material, inexpensive, simple and requires little chemical training. This test is a convenient probe for preliminary assessment of toxicity of natural products from plant biodiversity, heavy metals and pesticides (Aseer *et al.*, 2009).

2.9. Systematics of the collected antimalarial plant species and their economic importance

2.9.1. *Adansonia digitata* L. (Bombacaceae).

A. digitata is commonly known as Baobab. It is a massive deciduous tree indigenous to Africa with huge bark and white flowers. It is regarded as the largest succulent plant in the world (Kamatou *et al.*, 2011). The plant is restricted to hot, semi-arid regions and dry woodlands and it is found in Savannah woodlands of Sub-Saharan Africa. Baobab fruits, leaves, and bark are sources of food, fiber and medicine. Kamatou *et al.*, (2011) points out that over three hundred traditional uses of the plant have been documented. Due to its nutritional, medicinal and cosmetic uses, the plant has attracted the interest of many pharmaceutical industries (Gebauer *et al.*, 2002).

Fruit pulp, seeds and leaves of the plant are eaten because they are a good source of vitamins, proteins, carbohydrates and lipids (Sidibe & Williams, 2002). The plant's stem bark and leaves

are used in folk medicine to treat malaria (Gbadamosi *et al.*, 2011; Kamatou *et al.*, 2011). The plant has antipyretic, antimicrobial, anti-inflammatory and analgesic activities (Ramadan *et al*, 1994). Powdered leaves have anti-asthmatic and have anti-tension and antihistamine properties. Leaves are also used for other conditions such as diarrhea, dysentery, opthalmia and otitis media. Bark is used as a substitute for quinine to curb high fever and act as prophylactic measure for malaria as well (Sidibe & Williams, 2002).

Compaoré *et al.* (2011) has shown that flavonoids, proanthocyanidins and phenolic compounds are present in the pulp of *A. digitata* which make it a good radical scavenger. Elsewhere the fruits of *A.digitata* have been reported to contain proanthocyanidins as the major compounds (Shahat, 2006). Triterpenes, alkaloids, anthraquinones, saponins, tannins have also been reported to be present in the fruit pulp of *A.digitata* (Ramadan *et al.*, 1994: Gbadamosi *et al.*, 2011).

Figure 1: *A.digitata* tree

2.9.2. *Canthium glaucum* Hiern. (Rubiaceae)

The plant has been reported to be used traditionally for the treatment of malaria. A fruit decoction from the plant is taken orally by people of Msambweni district, Coast province; Kenya for the treatment of malaria (Nguta *et al.*, 2010). No bioactive compounds of *C.glaucum* have so far been reported. Root powder of a related species; *C. parviflorum* Lam. is used as a treatment for snake bites as reported by Mahishi *et al.* (2005). A powder from the same plant is used for dressing wounds and bone injuries (Ghorbani *et al.*, 2011). *C. dicoccum* (Gaertn.) Merr. a related species has been shown to contain sesquiterpenoids, nitrogenous compounds, aldehydes, terpinolene and phenols. These phytochemical constituents from extracts of *C.dicoccum* make the plant to have antimicrobial, anti-tumor, immunomodulatory and antioxidant properties (Raja *et al.*, 2011).

Figure 2: *C.glaucum* tree

2.9.3. *Launea cornuta* (Hocht. ex Oliv. & Hern) C.Jeffrey (Compositae)

L. cornuta is a perennial herb with erect stems which branch above to from a rosette of leaves and diffuse inflorescence. It grows in disturbed and artificial grasslands and roadsides. This is a vegetable used locally as a cheap source of food by the local people. It is an important plant species and is favored by most people because it has a good taste when cooked. Normally the fresh young leaves and shoots are plucked and cooked (Kataariina, 2000). Studies have shown that the plant contains high quantities of Vitamin C, Minerals like sodium, potassium, calcium and iron (Katariina, 2000). Likewise Lyimo *et al.* (2003) while investigating the nutrient composition of *L. cornuta*, they found that the leafy vegetable had a considerable amount of vitamin C, crude fiber, protein, fat, calcium and iron.

L.cornuta has been used locally for the management of prostate and breast cancer as reported by Kareru *et al.* (2007). The whole plant is crushed and the decoction obtained after boiling the crushed plant in water is given to the cancerous patient. Apart from cancer the plant species has been used as a remedy for diabetes, the whole plant is boiled and the decoction is drunk to reduce sugar levels in the body (Kareru *et al.*, 2007). On the health aspect, leaves and roots of *L. cornuta* are used medicinally in the management of malaria, stomach ache and in men they are used to stop blood flow during circumcision. *Launaea* species are antimicrobial and *L. cornuta* is one of the medicinal plants used by people of Suba district-Kenya particularly people living with HIV/AIDS to cure opportunistic diseases arising due to HIV/AIDS or to treat HIV/AIDS related symptoms (Nagata *et al.*, 2011).

L.nudicaulis (Linn.) Hook. has insecticidal activity against *Rhizopertha dominica* Fab. (Bostrichidae) and *Tribolium casteneum* Herbst. (Tenebrionidae); it has antifungal activity against *Microsporum canis* Bodin. (Arthrodermataceae) and *Aspergilus flavus* Joh.

(Trichocomaceae) and phytotoxic activity against *Lemma acquinoctialis* Welwitsch (Lemnaceae) (Ali *et al.*, 2003). *L. intybacea* (Jacq.) Beauverd, *L.massaiensis* (Fresen) Sch-Bip have hepatoprotective activity against hepatotoxins such as carbon tetrachloride (Chaudhary *et al.*, 2010). *L.procumbens* (Roxb.) Ram. & Raj. is antimicrobial, has cytotoxicity against brine shrimp *nauplii*, is antitumor and has antioxidant activity against a variety of radicals in the body, lastly it has hepatoprotective activity as has been demonstrated using rats by Rhamat (2010).

L.cassiniana Kuntze also has nematocidal activity against a number of soil nematodes. *L.intybacea* (Jacq.) Beauverd likewise was found to have hepatoprotective activity on paracetamol introduced hepatotoxicity in albino rats (Takate *et al.*, 2010). *L.sonchoides* (Cass.) N.Killian is antifungal and will inhibit the growth of *Trichoderma hamatum* (Bonord.) Bainier (Hypocreaceae) and *T.viridae* J.P.H van Wyk (Hypocreaceae) as proved by studies done by Abou-Zeid *et al.*, (2008). *Launaea* genus is characterized by flavonoids, triterpenes, sesquiterpene lactones, coumarins and steroids. Flavones like apigenin and luteolin are common compounds in the genus. Steroids like stigmasterol, cholesterol, taraxasterol have been identified in *L. nudicaulis*. Flavone glycosides such as apigenin-7-glycoside, luteolin-7-glycoside, luteolin-7-rutinoside and Vitexin have been isolated from the extract of *L.tenuiloba* (L.) Kuntze and *L.resedifolia* (L.) Kuntze. Delphinidin; an anthocyanin is found in *L.asplenifolia* Hook. (Ali *et al.*, 2003; Fairouz *et al.*, 2010).

Various species of *Launaea* such as *L.arborescens* (Batt.) Murb., *L.mucronata* (Forssk.) Muschl, *L.nudicaulis* and *L.capitata* (Spreng.) Dandy contains various types of flavones such as luteolin, apigenin and flavone glycosides such as apigenin 7-O-glucoside, vitexin, luteolin 7-O-glucoside and luteolin 7-O-rhamnoside besides others (Vipaporn & Christian, 2010). Two isoprenylated flavonoids; asplenitin and asplenetin 5-O-neohesperidoside have been identified in *L.*

asplenifolia (Denis & Ragaj, 1996). Roots of *L. mucronata* (Forssk.) Muschl, aerial parts of *L. spinosa* (Forssk.) Sch and aerial parts and roots of *L.tenuiloba* contain lactucin type guianolides as stated by Christian (2008)

Figure 3: Flowering *L.cornuta* plant

2.9.4. *Zanthoxylum chalybeum* Engl. (Rutaceae)

Z. chalybeum is a deciduous tree which reaches a height of 12 meters. It has pale grey bark with dark smooth scales and prickles and the crown is rounded. The tree grows well in low altitude areas, in dry woodlands and in savannah grasslands. The leaves when dried can be brewed to make a kind of a tea. Leaves are used as fodder and are fed on by goats while the tree trunk is a good source of charcoal and durable timber (Bamford & Henderson, 2003).

Stem, root bark and leaves are traditionally used for malaria (Beetje, 1994; Kokwaro, 2009). Roots and leave concoction is used in the management of chronic joint pains (Wambugu *et al.*,

2011). Seed extracts of *Z. chalybeum* has antiviral activity against measles virus (Olila *et al.*, 2002). It is active against *Staphylococcus aureus* Rosenbach. (Staphylococcaceae) and has anti-inflammatory activities (Matu & Staden *et al.*, 2003). The plant has been used traditionally in the management of diabetes (Keter & Mutiso, 2011).

Z.chalybeum has been reported to contain alkaloids which have antibacterial and cytotoxic activity (Chrian *et al.*, 2011). Preliminary screening of *Z. armatum* DC. has revealed that the plant contains linalool as the major essential oil (Tiwary *et al.*, 2007). *Z. schinifolium* Siebold & Zucc contains linalool and estragole as the main essential oils besides *ar*-tumerone and limonene. (Cheng *et al.*, 2011). *Zanthoxylum* species contains various compounds such as alkaloids, aliphatic and aromatic amides, lignans, coumarins, sesquiterpene lactones and sterols (Cheng *et al.*, 2011; He *et al.*, 2002). The compounds have cytotoxic, molluscicidal, anticonvulsant, anti-sickling, anaesthetic, antibacterial, anti-hypertensive and anti-inflammatory activities (Adesina, 2005).

Figure 4: *Z.chalybeum* tree

2.10. PROBLEM STATEMENT

Malaria is a killer disease responsible for many deaths annually and in Kenya 25 million are at risk of malaria, it is expensive to treat and manage and accounts for great loss in Gross Domestic Product particularly in developing countries due to its morbidity. Nowadays there is great urge to find alternative drugs to modern malaria medicines which are expensive and which may not be effective since malaria parasites are always mutating and becoming resistant to current malaria drugs. The search for antimalarials from plant biodiversity is one move to find alternative cheap medicaments to modern malaria medicine. The present study was conducted to evaluate the antimalarial activity, toxicity and phytochemical composition of selected anti-malarial plants commonly used by the Msambweni community to treat malaria.

2.11. JUSTIFICATION OF THE STUDY

In Kenya malaria causes great morbidity and mortality which is a drawback to economic development. Antimalarial drugs are expensive to poor communities in developing countries and human malaria parasites have developed resistance to almost all current antimalarial drugs. Nowadays researchers are trying to develop new drugs for malaria. Most modern medicines were discovered through study of plants which were used traditionally to treat specific illnesses. In addition, very few medicinal plants have been analyzed chemically and their bioactive constituents are yet to be validated. As a result, knowledge from traditional medicine can be very essential in the development of cheap and effective antimalarial drugs.

2.12. HYPOTHESIS

H_O:

Crude extracts of plants under investigation will not inhibit growth of *Plasmodium berghei* merozoites in mice and will not be toxic to *Artemia salina* L. nauplii.

H_A:

Crude extracts of plants under investigation will inhibit growth of *Plasmodium berghei* merozoites in mice and will be toxic to *Artemia salina* L. nauplii.

2.13. RESEARCH OBJECTIVES

2.13.1. General objective

To investigate *in vivo* anti-malarial activity, toxicity and phytochemical screening of *A.digitata, C.glaucum, L.cornuta* and *Z.chalybeum* which have been used in traditional medicine for the treatment of malaria.

2.13.2. Specific objectives

1. To determine *in vivo* antimalarial activity of organic [MeOH: $CHCl_3$ (1:1)] and aqueous extracts of *A.digitata, C.glaucum, L.cornuta* and *Z.chalybeum* against *P.berghei* merozoites.
2. To estimate acute toxicity (LD_{50}) of *A.digitata, C.glaucum, L.cornuta* and *Z.chalybeum* crude extracts using Brine shrimp (*Artemia salina L.*) nauplii
3. To analyse the phytochemical constituents in *A.digitata, C.glaucum, L.cornuta* and *Z.chalybeum* crude extracts using Thin Layer Chromatography (TLC)

3. CHAPTER THREE: MATERIALS AND METHODS

3.1. Collection of plant material

Plant parts were collected from Msambweni district, Coast province, Kenya. Collection was based on ethnopharmacological use as antimalarials through interviews with local communities (Nguta *et al.*, 2010). Information gathered included vernacular names (in parantheses) and the parts used in preparation of the herbal antimalarial remedies: *A. digitata* (*Mbamburi*) stem bark, *C. glaucum* (*Mhonga*) roots, *L. cornuta* (*Mtsunga wa utsunga*) leaves and *Z. chalybeum* (*Mjafari/mporojo*) stem bark.Plants were identified by a taxonomist from the University of Nairobi and voucher specimens were deposited in the University of Nairobi Herbarium. The plant parts were air dried under a shade, chopped into small pieces and then ground into powder. The collected plants are tabulated in Table 1.

Table 1: Plant species collected from Msambweni district based on their use as antimalarials

Plant Species/Family	**Plant Part Collected**	**Voucher Number**
Adansonia digitata L.(Bombacaceae)	Stem Bark	JN414
Canthium glaucum Hiern. (Rubiaceae)	Roots	JN426
Launaea cornuta (Hocht.ex.Oliv. & Hiern.) C.Jeffrey (Compositae)	Leaves	JN028
*Zanthoxylum chalybeum.*Engl. (Rutaceae)	Stem Bark	JN040

3.2. Preparation of crude extracts

For each plant, 50 grams of the ground plant material was extracted with 500 mls of distilled water while another 50 grams was extracted with 500 mls of chloroform-methanol mixture (1:1 v/v) for four times at 48 hour intervals using cold maceration (Sulsen *et al.*, 2011). The aqueous extracts were filtered and the filtrate kept in a deep freezer then lyophilized resulting to a dry gummy substance. Organic ($CHCl_3$: MeOH) extracts were filtered and concentrated with a rotary

evaporator then left to dry powder. The dry solid extracts were stored at -20^{0}C in air tight containers until used. The yields of the extracts are shown in Table 2.

Table 2: Yield in grams after extraction of ground plant material with water and a mixture of chloroform-methanol (1:1)

Plant species/Family	**Aqueous extracts (Grams)**	**Organic ($CHCl_3$:MeOH) extracts (Grams)**
Adansonia digitata L. (Bombacaceae)	7.58	4.13
Canthium glaucum Hiern. (Rubiaceae)	9.52	4.40
Launaea cornuta (Hocht.ex.Oliv.& Hiern) C.Jeffrey (Compositae)	8.12	5.60
Zanthoxylum chalybeum.Engl. (Rutaceae)	16.02	6.48

3.3. *In vivo* determination of anti-malarial activity

In vivo anti-malarial activity was determined by 4-day suppressive anti-malarial assay according to Waako *et al.* (2005) and Peters *et al.* (1975). Cryopreserved chloroquine sensitive *Plasmodium berghei* (ANKA strain) parasites were obtained from KEMRI where they were stored at -80^{0}C. For the parasites to be used for experimental purposes, they were revived and stabilized in mouse host according to Ravindran *et al.* (1982). Mice were housed in cages and given mice pellets and water *ad libitum*. A group of three naive mice were used to revive and stabilize the *P.berghei* parasites. Reviving, stabilizing and mantainance of the *Plasmodium berghei* parasite was achieved by continuous reinjection of the parasite to new naive mice since infected mice died after several days due to increased parasitaemia. One mouse was chosen as a donor mouse which was then anesthetized using chloroform in a fume chamber and blood collected via cardiac puncture into heparinized bottles to make innoculum for infecting new naive mice. After drawing blood, the donor mouse was sacrificed by cervical dislocation. Even after the parasite had stabilized in the mouse host, passing the parasite to new mice ensured that stabilized parasites

were always available for experimental purposes. Normally, passages were done when the parasitaemia reached about 40% which reached that point by the fourth day once the parasites were introduced to naive mice. As a result, the process of passing parasites from donor mice to naive mice was followed after every 4 days. Three passages were ideal to stabilize cryopreserved parasite in the mouse host.

Appropriate innoculum for infecting mice had low parasitaemia of around 1% and experimental mice were innoculated with 0.2 mls of blood with 1% parasitaemia (containing about 1.0×10^7 parasitized cells) according to Waako *et al.*, (2005). Before infecting experimental mice, parasitaemia in the donor mice was first determined in order to dilute the blood to achieve 1% parasitaemia with Phosphate Buffered Saline (PBS). Percentage parasitaemia of the donor mice was calculated as follows:

$$\textbf{Percentage Parasitaemia} = \frac{\textbf{Total Number of Parasitized Cells}}{\textbf{Total Number of Cells}} \times \textbf{100}$$

Table 3: Determination of parasitaemia of the donor mouse

Donor Mouse 1	**Four magnification fields (MFs)**				**Total**	**parasitaemia**
Parasitized Cells	92	101	91	82	**366**	**73.79%**
Total Cells	135	132	123	106	**496**	
Donor Mouse 2						
Parasitized Cells	69	78	76	95	**316**	**44.40%**
Total Cells	175	136	237	156	**704**	

Donor Mouse 2 which had a parasitaemia of 44.40% was selected as the donor mice because it had a high parasitaemia of > 20%.The reason why donor mouse 1 was not chosen was that it was very weak during parasitaemia estimation and dead before parasite harvest because of the extreme high parasitaemia. The parasitaemia was reduced to 1% by dilution using Phosphate

Buffered Saline (PBS). 9.77 mls of PBS were required to dilute 0.23 mls of blood from the donor mice with 44.40% parasitaemia to 10 mls of blood with 1% parasitaemia which was used in infecting 50 experimental mice each receiving 0.2 mls of the diluted blood through intraperitoneal route containing approximately 1.0×10^7 parasitized cells. Swiss albino mice [*Mus musculus* L.(Muridae)] irrespective of sex about 8 weeks old weighing between 18-22 grams (approximately 20 grams) were then infected with the *Plasmodium berghei* parasites and then kept in the main chamber before they were divided into groups of five.

A completely randomized design was employed in conducting the experiment. 10 cages of mice were selected and in each cage 5 infected mice were assigned randomly from the main chamber. Mice in each cage were numbered (by marking on the tails using a pelt pen) for easy identification during administration of plant extracts and preparation of blood smears. The cages were assigned treatments randomly. Mice in four cages received oral administration of aqueous extracts at a dosage rate of 100 mg/kg/day for four days. Another group of mice in four cages received oral administration of $CHCl_3$: MeOH extracts at a dosage rate of 100 mg/kg/day for four days. The last two cages were used as control cages; one positive control and one negative control. The experimental design is summarized in Table 4.

Table 4: Random allocation of infected mice

	TREATMENTS									
	Aqueous extracts				Organic ($CHCl_3$:MeOH) extracts				Controls	
DAYS	A	B	C	D	A1	B2	C2	D2	+Ve	-Ve
Day 0	Cage 1	Cage 3	Cage 7	Cage 2	Cage 10	Cage 4	Cage 6	Cage 9	Cage 5	Cage 8
Day 1	Cage 1	Cage 3	Cage 7	Cage 2	Cage 10	Cage 4	Cage 6	Cage 9	Cage 5	Cage 8
Day 2	Cage 1	Cage 3	Cage 7	Cage 2	Cage 10	Cage 4	Cage 6	Cage 9	Cage 5	Cage 8
Day 3	Cage 1	Cage 3	Cage 7	Cage 2	Cage 10	Cage 4	Cage 6	Cage 9	Cage 5	Cage 8
Day 4	**Preparation of thin blood smears**									

Key: A, B, C, D=Treatments, +Ve = Positive, -Ve =Negative

Stock solutions of aqueous extracts (10,000 µg/ml) were made in distilled deionized water and filter sterilized using 0.22 µm membrane filters in a laminar flow hood. To make stock solutions of the $CHCl_3$: MeOH extracts, the organic extracts were dissolved in dimethylsulphoxide (DMSO) followed by subsequent dilution to lower concentration of DMSO to < 1% to avoid carry over (solvent) effect. Plant extracts were administered once daily orally (D0 to D3) at a dosage rate of 100 mg/kg in a dose volume of 0.2 mls. Positive control drug used was chloroquine (20 mg/kg/day) while negative control group was treated with distilled water (0.2 mls/mice/day). On the first day; plant extracts were administered 2 hours after infection of the mice with the parasites

Blood was obtained from each of the experimental mice on Day 4. The tip of the tail was cut with a sterilized scissors and blood was squeezed from the tail and thin blood smears were prepared. Forty eight (48) thin blood smears were prepared from the forty eight surviving mice on Day 4. Smears were fixed with methanol for 5 minutes and stained with 10% Geimsa which was prepared by mixing 100% Geimsa stain with staining buffer in the ratio 1:9.

The slides were observed under compound microscope under oil immersion at ×1000 to determine the number of parasitized cell per given magnification field (MF). For each blood smear specific for a given mouse, four magnification fields were observed and the number of parasitized cells (schizonts) and the total number of cells in the magnification field were recorded. The data obtained was used to determine percentage parasitaemia and parasite growth inhibition in each mouse. The number of dead mice was also recorded every day since the start of the four day suppressive test for the next 10 days.

3.4 Brine shrimp toxicity test

Brine shrimp-Leech (*Artemia salina L.*) larvae were used to determine the toxicity of the crude extracts according to Wanyoike *et al.*, (2004). Brine shrimp eggs were hatched in a shallow rectangular hatching tank containing artificial sea water and yeast .Yeast was provided to act acts as food for the hatching larvae. Hatching tank measured 14 cm by 9 cm by 5 cm and had a capacity of 225 mls. The tank had two compartments; one small and one large compartment. These two unequal compartments were created by a plastic divider with several 2 mm holes which allow hatched Brine shrimp larvae to pass through.

Artificial sea water or 3.3% saline was prepared by dissolving 33.04 grams of commercial sea salt in one liter of distilled water. 200 mls of artificial sea water was poured into the hatching tank to fill it to the brim. This was followed by pouring three dosage spoons of *Artemia salina* eggs and one gram of yeast to the larger compartment of the hatching tank. The larger compartment was then covered to prevent light exposure because Brine shrimp eggs hatch in dark while the smaller compartment was exposed to light. Hatching occurred within 36 to 48 hrs at 26^0C. Once the Brine shrimp larvae hatched, they moved from the larger compartment to the

smaller compartment through the 2 mm holes in the plastic divider where they were collected and transferred to fresh artificial sea water.

Various concentrations of the crude extract in sea water were used: 10,100 and 1000 µg/ml. A stock solution for each crude extract of10,000 µg/ml was initially prepared from which serial dilutions were carried out to make the three concentrations which were used in the experiment. For the aqueous extracts, the stock solution of 10,000 µg/ml was prepared by dissolving 0.1g of each crude extract in 10 mls of distilled water. For the $CHCl_3$: MeOH extracts, 0.1g of each sample was first dissolved in dimethylsulphoxide (DMSO) then diluted further using artificial water to 10 mls to make stock solution of 10,000 µg/ml. DMSO content in the stock solution was less than 0.05% to avoid DMSO carry over effect on Brine shrimp larvae. The stock solutions from various crude extracts were filtered using 0.22 µm micro filters under lamina flow hood (Wanyoike *et al.*, 2004).

Using Pasteur pipettes, 10 Brine shrimp larvae were transferred from the smaller compartment of the hatching tank to plastic tubes. The volume of artificial sea water in each plastic tube containing 10 Brine shrimp larvae was increased to 5ml except the tubes for 1,000 µg/ml which were topped to 4.5 mls with artificial sea water. Using micropipettes, 0.5 mls, 0.05 mls and 0.005 mls were transferred from the stock solution to the plastic tubes containing 5mls artificial sea water to make experimental solutions containing 1000 µg/ml, 100 µg/ml and 10 µg/ml respectively. A control group containing artificial sea water and Brine shrimp larvae only was included in the experiment and this denoted a concentration of 0 µg/ml. For each crude extract at each concentration three repeats were carried out. Survivors were counted after 24 hours using a magnifying glass (Meyer *et al.*, 1982). For each crude extract, experimental set up in Table 5 was followed.

Table 5: Brine shrimp experimental set up

Volume (ml) of artificial sea water	Brine shrimp larvae	Stock solution volume (ml)	Concentration (µg/ml)	Experiment type	Final volume in the tube (mls)
4.5	10	0.5	1,000	Trial(T)	5
4.5	10	0.5	1,000	Repeat(R)	5
4.5	10	0.5	1,000	Repeat(R)	5
5	10	0.05	100	T	5
5	10	0.05	100	R	5
5	10	0.05	100	R	5
5	10	0.005	10	T	5
5	10	0.005	10	R	5
5	10	0.005	10	R	5
5	10	0	0	1st Control	5
5	10	0	0	2nd Control	5
5	10	0	0	3rd Control	5

3.5. Phytochemical screening for secondary metabolites using Thin Layer Chromatography (TLC)

Crude plant extracts were screened for alkaloids, flavonoids, saponins and sesquiterpene lactones according to procedures described by Harborne (2002) with minor modifications. Extracts were first dissolved in chloroform and methanol (1:1). Aluminium TLC plates measuring 6.5 cm by 5 cm were used in phytochemical screening. Using micropipettes, about 2 mm diameter spots of the dissolved extracts were made on the plates along the origin. Origin was 1 cm from the base of the plates while solvent front was 5 cm from the base of the plates. Plates were put in a chamber containing appropriate chromatographic solvent system specific for the determination of the presence of a given class of phytochemical constituents. Details of phytochemical screening for alkaloids, flavonoids, saponins and sesquiterpene lactones are tabulated in Table 6.

Table 6: Screening for alkaloids, flavonoids, saponins and sesquiterpene lactones

Class of Secondary metabolites	Solvent system	Detection
Alkaloids	Dichloromethane: Methanol (85:15) (Harborne, 2002)	Dragendorff's reagent was sprayed on the developed plates. Formation of orange colors indicated the presence of alkaloids. Spraying the plates with sodium nitrate made the orange colors more intense (Harborne, 2002)
Flavonoids	n-hexane: ethyl acetate: acetic acid (6:3:1) (Waksmundzka *et al.*2008)	At 254 nm, flavonoids appeared as dark blue zones on a yellow background on the developed plates. The intensity of the yellow color in the background increased when ammonia was sprayed on the plates. At 365 nm flavonoids fluoresced yellow, blue or green (Waksmundzka *et al.* 2008).
Saponins	Dichloromethane: Ethyl acetate (9:1) according to Karem *et al.*(2005)	Detection of saponins occurred when plates were sprayed with a mixture of ethanol and H_2SO_4 (9:1) and then heated at 110^0C for 10 minutes. Saponins appeared as black spots (Karem *et al.*, 2005)
Sesquiterpene lactones	n- hexane: ethyl acetate (9:1) (Waksmundzka *et al.*, 2008).	Detection was done by placing the developed plates in a chamber containing iodine crystals to observe brown spots (Waksmundzka *et al.*, 2008). Sesquiterpene lactones were also detected as brown, yellow spots when plates were sprayed with concentrated H_2SO_4 and heated for 5 minutes at 100-110^0C (Harborne, 2002).

3.6. Statistical analysis

Parasitaemia inhibition was analyzed using SPSS Version 16. One way ANOVA was used to analyze chemosuppression means obtained from the four day suppressive assay to determine whether chemosuppression caused by one plant extract was different from chemosuppressions

caused by the other plants extracts according to Morgan *et al.*, (2004). Once the means were found to be different from each other, Dunnett test was then used for multiple comparisons of chemosuppressions to determine whether chemosuppressions arising from the various treatments were different from the chemosuppression induced by chloroquine (positive control). The significance level used in the analysis was 0.05 (Alpha Level $\leq$ 0.05).

Brine shrimp toxicity was determined by probit analysis. Probit analysis is a specialized regression model of binomial response variable and it has been used to analyse dose-response experiments in a variety of fields. It involves conversion of concentrations into logarithms and the corresponding percentage mortalities into probits. The logarithms of the concentrations are then plotted against the probits to give a straight line graph. This regression line is then used in the determination of LD_{50} (Ashford & Sowden, 1970).

4. CHAPTER FOUR: RESULTS

4.1. Calculation of parasitaemia and parasitaemia inhibition

After counting the total number of parasitized cells and the total number of cells in four magnification fields (MFs) in each microscope slide, mean parasitaemia and chemosuppression in each mouse was determined. Percentage parasitaemia and chemosuppression was determined using the formula below which was described by Hilou *et al.* (2006)

$$\textbf{Percentage Parasitaemia} = \frac{\textbf{Total Number of Parasitized Cells}}{\textbf{Total Number of Cells}} \times 100$$

$$\textbf{Chemosuppression} = \frac{(\textbf{Parasitaemia in the negative control}) - (\textbf{Parasitaemia with drug})}{\textbf{Parasitaemia in the negative control}} \times 100$$

Table 7 and Table 8 shows the percentage parasitaemia and percentage chemosupppression in each mouse in all the 10 treatments used in the experiment (four aqueous extracts, four $CHCl_3$: MeOH extracts and two controls). See Appendix 1 for more details about calculation of percentage parasitaemia and chemosuppression of each mouse.

Table 7: Percentage parasitaemia and chemosuppression in the groups treated with aqueous extracts at a dosage of 100 mg/kg per mice for 4 days.

	Aqueous extracts											
Treatments	*Adansonia digitata*		*Canthium glaucum*		*Launaea cornuta*		*Zanthoxylum chalybeum*		Negative control(DW)		Positive control(CQ)	
Percentage	P	C	P	C	P	C	P	C	P	C	P	C
Mice1	22.68	28.86	27.50	13.74	21.70	31.95	9.51	70.19	33.73	N/A	4.45	86.04
Mice2	6.08	80.92	22.05	30.83	19.65	38.37	12.67	60.27	33.41	N/A	1.18	96.30
Mice 3	9.05	71.63	26.25	17.66	16.12	49.45	29.16	8.53	28.42	N/A	3.93	87.69
Mice 4	-	-	17.87	43.95	21.48	32.64	13.87	56.51	30.06	N/A	6.63	79.21
Mice 5	-	-	14.75	53.73	19.69	38.23	22.59	29.14	33.80	N/A	4.17	86.92
Average P & C	12.60	60.47	21.68	31.98	19.72	38.13	17.56	44.93	31.88	0	4.07	87.23

Key: DW=Distilled water, CQ=Chloroquine, P= Percentage parasitaemia, C= percentage chemosuppression.

Table 8: Percentage parasitaemia and chemossuppresion in the groups treated with $CHCl_3$: MeOH (1:1) extracts at a dosage of 100 mg/kg per mice for 4 days

	Organic($CHCl_3$:MeOH) extracts											
Treatments	*Adansonia digitata*		*Canthium glaucum*		*Launaea cornuta*		*Zanthoxylum chalybeum*		Negative control(DW)		Positive control(CQ)	
Percentage	P	C	P	C	P	C	P	C	P	C	P	C
Mice1	26.96	15.43	21.63	32.15	30.18	5.33	22.69	28.83	33.73	N/A	4.45	86.04
Mice2	24.53	23.06	22.56	29.23	1.96	93.85	20.53	35.60	33.41	N/A	1.18	96.30
Mice 3	30.54	4.20	17.22	45.98	24.83	22.11	21.10	33.81	28.42	N/A	3.93	87.69
Mice 4	20.75	34.91	8.65	72.87	22.88	28.23	24.19	24.12	30.06	N/A	6.63	79.21
Mice 5	4.17	86.92	19.58	38.58	30.07	5.68	26.96	15.53	33.80	N/A	4.17	86.92
Average P & C	21.39	32.90	17.93	43.76	21.98	31.04	23.09	27.56	31.88	0	4.07	87.23

Key: DW=Distilled water, CQ=Chloroquine, P= Percentage parasitaemia, C= percentage chemosuppression.

Table 9 shows the mean parasite density, chemosuppression and survival time of *P.berghei* infected mice treated intraperitoneally with aqueous and organic extracts at a dose of 100 mg/kg body weight, once a day for four days.

Table 9: Mean parasite density, chemosuppression and survival time of *P.berghei* infected mice

ueous extracts				
nt species/Family	Voucher specimen number	% mean(X±SEM) parasite density	% mean(X±SEM) chemosuppression	% mice survival by day 10
itata L.(Bombacaceae)	JN414	12.60±5.11	60.47±16.03	20
laucum Hiern. (Rubiaceae)	JN426	21.68±2.42	31.98±7.60	20
rnuta (Hocht.ex.Oliv. & Hiern) Jeffrey(Compositae)	JN028	19.72±0.99	38.13±3.13	0
halybeum.Engl. (Rutaceae)	JN040	17.56±3.62	44.93±11.36	20
nole($CHCl_3$:MeOH) extracts				
itata L.(Bombacaceae)	JN414	21.39±4.59	32.90±14.40	0
laucum Hiern. (Rubiaceae)	JN426	17.92±2.49	43.76±7.83	20
rnuta (Hocht.ex.Oliv. & Hiern) Jeffrey(Compositae)	JN028	21.98±5.21	31.04±16.33	0
alybeum.Engl. (Rutaceae)	JN040	23.09±1.16	27.56±3.635	0
ntrols				
tive control (Distilled water)		31.88±1.11	0	0
itive control (Chloroquine)		4.07±0.81	87.23±2.72	100

P<0.05, S.D=Standard error of the mean

4.2. Mean plots

Figures 5 shows parasitaemia and chemosuppressions induced by various plant extracts. Both parasitaemia and chemosuppesssion are dependent on the treatments, low parasitaemia means treatment is working while chemosuppression provides the extent of extract activity.

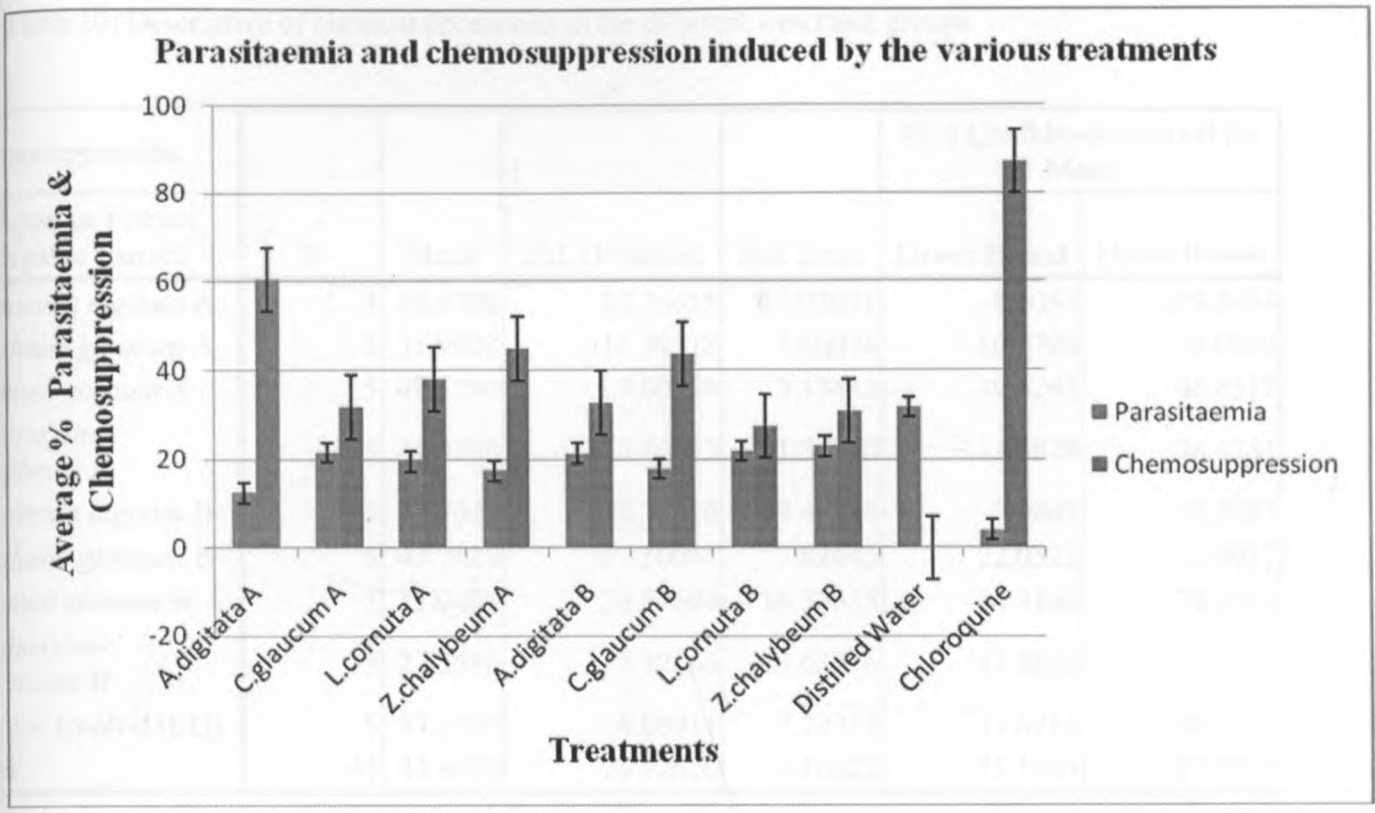

Figure 5: Parasitaemia and chemosuppressions elicited by various plant extracts

Key: A=Aquoeus extract, B=Organic ($CHCl_3$: MeOH) extract

Plant extracts which induced relatively high chemosuppression compared to the other plant extracts included aqueous extracts of *A.digitata, L.cornuta* and *Z.chalybeum* with chemosuppression of 60.47%, 38.13% and 44.93% respectively. $CHCl_3$: MeOH extracts of *C.glaucum* also had a chemosuppression of 43.76%. The antimalarial drug chloroquine which was used in the experiment induced the highest chemosuppression of 87.23%.

Table 10: Descriptive of chemosuppressions in the different treatment groups

Chemosuppression					95% Confidence Interval for Mean	
A=Aqueous Extract O=Organic Extract	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound
Adansonia digitata A	3	60.4700	27.76635	16.03091	-8.5054	129.4454
Canthium glaucum A	5	31.9820	16.99578	7.60074	10.8790	53.0850
Launaea cornuta A	5	38.1280	7.00968	3.13482	29.4243	46.8317
Zanthoxylum chalybeum A	5	44.9280	25.40553	11.36170	13.3829	76.4731
Adansonia digitata B	5	32.9040	32.20216	14.40124	-7.0803	72.8883
Canthium glaucum B	5	43.7620	17.50047	7.82645	22.0323	65.4917
Launaea cornuta B	5	31.0400	36.52694	16.33535	-14.3142	76.3942
Zanthoxylum chalybeum B	5	27.5580	8.12846	3.63516	17.4652	37.6508
Positive Control (CQ)	5	87.2320	6.08911	2.72313	79.6714	94.7926
Total	43	43.4670	26.92626	4.10622	35.1803	51.7537

A = Aqueous extract B = Organic extract

Table 11: ANOVA analysis

One Way ANOVA

Chemosuppression

Differences	Sum of Squares	Degrees of Freedom	Mean Square	F Statistic	Significance (Sig.)
Between Groups	13852.859	8	1731.607	3.547	.004
Within Groups	16598.122	34	488.180		
Total	30450.981	42			

Table 11 above shows the results of one way ANOVA. When the significance value obtained is less than 0.05, it denotes that there is significance difference between the means being compared (Morgan *et al.*, 2004). From the calculations, the F Statistic value obtained is 3.547 and its associated Level of Significance is 0.004 which is less than the required level of significance of

0.05. This implies that the chemosuppressions in the various treatments were significantly different from each other.

Multiple comparison of chemosuppressions show that the comparison of the chemosuppression induced by *A. digitata* aqueous extract and the chemosuppression induced by the positive control gave a significance value of 0.451. This significance level obtained of 0.451 is much larger than the required 0.05 significance level. This implies that there was no significance difference between chemosuppression induced by the *A. digitata* aqueous extract and the chemosuppression induced by chloroquine according to Morgan *et al.*, (2004). The comparisons of the other treatments with the positive control resulted to a significance value which is less than 0.05 and this implies that the chemosuppression induced by such treatments were different from the positive control chemosuppression (See Appendix 2)

4.3. Mice mortality

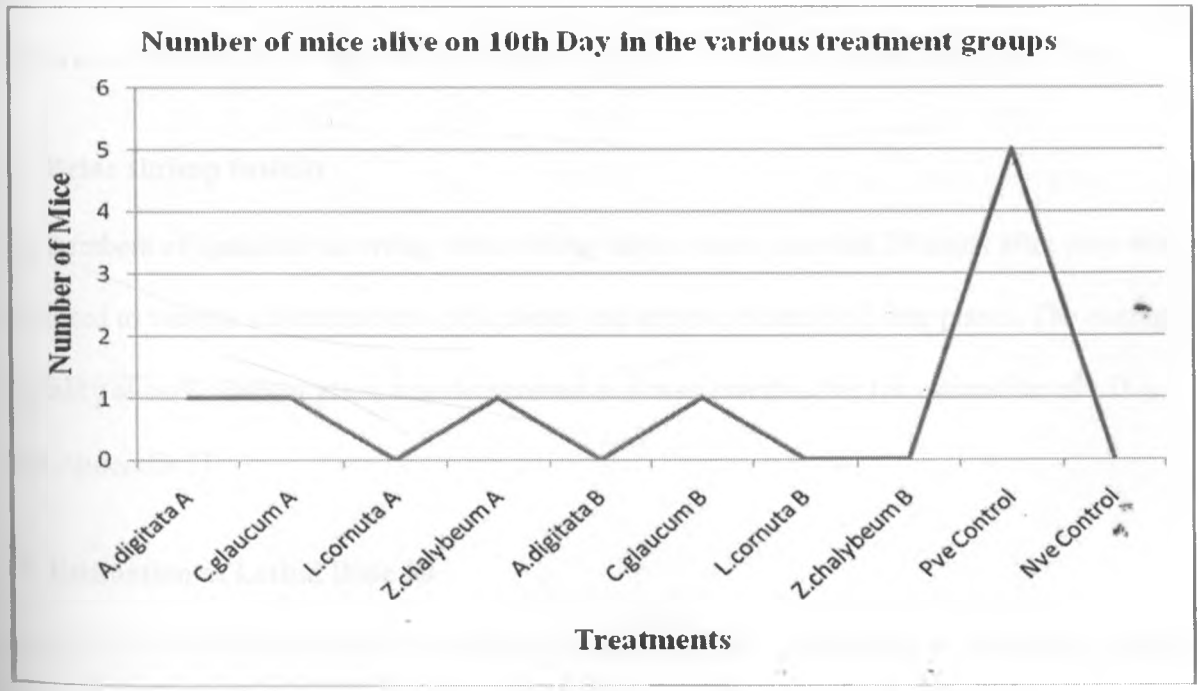

Figure 6: Number of Mice alive in each treatment group on the 10th Day

Key: A=Aqueous extract, B=Organic ($CHCl_3$: MeOH) extracts, Pve Control = Positive Control, Nve = Negative Control.

Since the start of the 4-day suppressive test, mortality of the mice was monitored on daily basis. Mice death occurred at different times and this could be attributed to the toxicity of the plant extracts administered, increased parasitaemia or intervening environmental factors. Figure 6 above shows the number of mice which died in each cage for a period of 10 days since the start of the 4-day suppressive antimalarial test.

The crude extracts administered to the mice were able to keep at least some of the mice alive up to the 10^{th} day. Groups treated with organic extracts of *C.glaucum* and aqueous extracts of *A.digitata, C.glaucum* and *Z.chalybeum* had mice alive on the 10^{th} day. No mice died in the positive control group which received oral administration of chloroquine on daily basis and all the mice were healthy in the 10^{th} day and were later sacrificed by cervical dislocation because they didn't die. In the negative control group which was receiving distilled water on daily basis, all the mice died by the 7^{th} day making it the first group to have 100% mortality by 7^{th} day.

4.4. Brine shrimp toxicity

The numbers of dead and surviving brine shrimp larvae were recorded 24 hours after they were subjected to various concentrations of aqueous and organic extracts of four plants. The average mortality at each concentration was determined as it was essential for the estimation of LD_{50} (See Appendix 3).

4.5. Estimation of Lethal Dose 50

Finney Probit analysis Program was used to estimate the LD_{50} of the plant extracts on the brine shrimp larvae according to Meyer et al., (1982). The average mortality in the three

concentrations (1000 µg/ml, 100 µg/ml and 10 µg/ml) for each plant was fed into the program to estimate the LD_{50}.The results are tabulated below.

Table 12: Acute toxicity of the crude plant extracts

	Aqueous extracts					
	Average mortality at various concentrations				LD_{50}	Gradient(G)
Plant species	1,000 µg/ml	100 µg/ml	10 µg/ml	0 µg/ml	µg/ml	
A.digitata L.	2.33	0.33	0	0	9563.59	1.8009
C.glaucum Hiern	5.33	0.33	0.66	0	1209.67	0.6930
L. cornuta (Hocht.ex.Oliv.)C.Jeffrey	8	1.33	0.33	0	341.64	0.4321
Z.chalybeum .Engl.	10	0.33	0	0	268.28	0.3247
$CHCl_3$:MeOH extracts						
A.digitata L.	10	3.33	0.66	0	110.23	0.3203
C.glaucum Hiern	2	0.66	0	0	1783.59	2.1700
L. cornuta (Hocht.ex.Oliv.)C.Jeffrey	10	4	2	0	75.26	0.3400
Z.chalybeum Engl.	10	9	2	0	25.78	0.4357

A plant extract with a LD_{50} ranging between 0-500 µg/ml implies that the extract is highly toxic while that ranging between 500-1000 µg/ml implies that the extract is moderately toxic. On the other hand a plant extract with a LD_{50} which is over 1000 µg/ml implies that the plant is non toxic (Nguta *et al.*, 2011).

From the experiment aqueous extracts of *A.digitata* stem bark and *C.glaucum* roots have LD_{50} which is over 1000 µg/ml and hence they are non toxic on Brine shrimp larvae. However crude extracts of *Z.chalybeum* stem bark and *L.cornuta* leaves have LD_{50} value which lies between 0 µg/ml and 500 µg/ml hence are highly toxic on Brine shrimp larvae

4.6. Phytochemical analysis of crude plant extracts for secondary metabolites

Crude extracts were screened for secondary metabolites using TLC and the results are tabulated in Table 13 and Table 14.

Table 13: Phytochemical screening of aqueous crude extracts

Aqueous extracts	**Alkaloids**	**Flavonoids**	**Sesquiterpene lactones**	**Saponins**
A.digitata L.	+ve	+ve	-ve	-ve
C.glaucum Hiern	+ve	+ve	-ve	-ve
L. cornuta (Hocht.ex.Oliv.)C.Jeffrey	+ve	+ve	-ve	+ve
Z.chalybeum Engl.	+ve	+ve	-ve	-ve

Key: +ve= Present, -ve= Absent

Table 14: Phytochemical screening of the crude $CHCl_3$: MeOH extracts

$CHCl_3$: MeOH extracts	**Alkaloids**	**Flavonoids**	**Sesquiterpene lactones**	**Saponins**
A.digitata L.	+ve	+ve	+ve	+ve
C.glaucum Hiern.	+ve	+ve	+ve	+ve
L. cornuta (Hocht.ex.Oliv.)C.Jeffrey	+ve	+ve	+ve	+ve
Z.chalybeum .Engl.	+ve	+ve	+ve	+ve

Key: +ve=Present, -ve=Absent

5. CHAPTER FIVE: DISCUSSION

Parasitaemia refers to the level of infection by a particular parasite or the number of parasitized cells circulating in blood. A decline in parasitaemia in a given organism is essential for the recovery of symptomatic malaria. Effective antimalarial drugs when given in correct dosage will usually lead to decrease in parasitaemia. When mice were infected with *Plasmodium* parasites, the parasites multiplied in the erythrocytes and later caused bursting of the erythrocytes releasing merozoites into circulation which attacked more erythrocytes causing increased parasitaemia.

The percentage parasitaemia in the negative control group on D4 after infection with *Plasmodium berghei* parasites was 31.88 % which was the peak parasitaemia witnessed in all the groups treated with various plant extracts. The other treatments had lower parasitaemia levels than this. Parasitaemia in the group which was treated with chloroquine was lowest (positive control group) which was 4.07 % (See Table 7). Parasitaemia in the negative control being higher than in the other treatment groups shows that the treatments had an effect on the growth of *Plasmodium berghei* parasites in mice.

Parasitaemia increased gradually and without any antimalarial drug being administered in the negative control all the mice died on the 7^{th} day. In the other treatment groups, most of the mice were alive by day seven post infection and even by the 10^{th} day some groups had mice which were alive. However no mice died in the positive control group and by 10^{th} day all the mice were alive and healthy (See Figure 6).

Drugs lead to decreased parasitaemia and subsequent recovery of symptomatic malaria. They also reduce parasitaemia through various ways like reducing parasite nutrient intake, interfering with parasite metabolic pathways like heme metabolic pathway which is involved in the metabolism of iron (De villers & Egon, 2009). Drugs also negatively affect parasite reproduction

and growth (Ziegler *et al.*, 2002). The plant extracts to some extend cleared the parasites and reduced the level of parasitaemia and made the mice to survive even up to the 10^{th} day. Chloroquine had a good chemosuppression of 87.23 % as determined on the fourth day post infection and a 100% survival rate by day ten post infection.

Chemosuppression is inversely related to parasitaemia and plant extracts which have been shown to reduce parasitaemia to low levels have been shown to have a corresponding high chemosuppression. The aqueous extracts of *A. digitata* had a chemosuppression of 60.47% ($p<0.05$), which was the peak chemosuppression recorded in the experiment apart from the chemosupression induced by chloroquine. Chemosuppression below 50% is usually taken as low since calculations are based on the negative control parasitaemia as baseline. Due to that, the other extracts had low chemosuppression which included the organic ($CHCl_3$: MeOH) extracts of *C.glaucum,* aqueous extracts of *Z. chalybeum* and aqueous extracts of *L. cornuta* with 43.76%, 44.93% and 38.13% respectively which their chemosuppression was slightly high among the extracts with low chemosuppression.

Aqueous extracts of *A.digitata* induced a chemosuppression which was not significantly different from the chemopsuppression induced by chloroquine. Although the other plants extracts induced chemosuppression which was significantly different from that induced by chloroquine, they should also not be ruled out because to some extent some of the mice in those treatments had a longer life span than the mice in the negative control group implying that the plant extracts had a significant antimalarial activity but only which could not match that of chloroquine

From the study, aqueous extracts from the stem bark of *A.digitata* exhibited high antimalarial activity. This observation supports earlier work by Ajaiyeoba (2005) who reported that methanolic extracts of the stem bark of *A. digitata* were able to reduce the number of

Plasmodium parasites in mice. Previous studies have shown that water and methanol extracts of the stem bark of *Z. chalybeum* have significant *in vitro* antimalararial activity against chloroquine sensitive and chloroquine resistance strains of *Plasmodium falciparum* (Rukunga *et al.*, 2009) which is in line with the results on *Z.chalybeum* obtained in the current study. Methanolic extracts of the root bark of *Z.chalybeum* have been reported to have significant antimalarial activity on *Plasmodium falciparum* as argued by Muganga *et al.* (2010). This is also in agreement with observations in the current study. Elsewhere aqueous extracts of a related species *Zanthoxylum usambarense* (Engl.) Kokwaro have also been shown to exhibit a significant antiplasmodial activity against *Plasmodium falciparum* (Kirira *et al.*, 2006). Although *C.glaucum, L.cornuta* are used in traditional medicine for treatment of malarial there is no previous information which has been reported on their *in vivo* antimalarial activities. Therefore the current study reports for the first time *in vivo* antimalarial activity of the two plant species.

The significant within group variation in the percentage chemosuppression in the groups treated with aqueous extracts of *A.digitata* and *Z.chalybeum* can be attributed to the fact that administration of plant extracts on mice was done orally. Due to this, some of the mice may have not taken the whole 100 mg/kg dosage and this may have affected the parasitaemia in those groups. Another factor which could account for the within group variation could be that the experimental mice were chosen irrespective of sex. Sex is a factor which affects many physiological reactions one of them being drug interactions. Female mice and male mice may have responded to the treatment differently and this may have brought the variation in chemosuppression within the given treatment groups. Lastly the fact that the experimental mice weighed between 18-22g could have brought about the within group variation because an

approximate weight of 20g was used and assigned to each mouse in the calculation of the required dosage of 100 mg/kg and the slight differences in mice weight were not accounted.

LD_{50} is the concentration of dose which kills fifty percent of all organisms in a given experiment. Aqueous extracts of both *A.digitata* and *C.glaucum* had a LD_{50} > 1000 µg/ml hence were considered to be non toxic to Brine shrimp larvae while aqueous and organic ($CHCl_3$: MeOH) extracts of *L.cornuta, Z.chalybeum* which had a LD_{50} < 500 µg/ml were classified as being toxic to Brine shrimp larvae with more toxicity being witnessed with the organic ($CHCl_3$: MeOH) extracts (See Table 12). *A.digitata* has also been shown to be non toxic on mice from previous studies. For instance LD_{50} of aqueous extract of the fruit pulp of *A.digitata* on mice was found to be over 8000 µg/ml (Ramadan *et al*., 1994). This justifies the results obtained in this study on the non toxicity of stem bark of *A.digitata* on Brine shrimp larvae. Non toxicity of *A.digitata* explains why most of the plant parts: seeds, fruit pulps and leaves are consumed by many communities. Nguta *et al.* (2011) while investigating toxicity of aqueous extracts of the leaves, stem bark and root bark of *Z.chalybeum* on Brine shrimp larvae obtained LD_{50}<500 µg/ml for the three plant parts which confirms the results obtained in this study implying that *Z.chalybeum* is a plant with high toxicity on Brine shrimp larvae and thus could not make safe antimalarial herbal remedies. This calls upon for dose adjustment amongst communities using this plant for preparation of antimalarial herbal decoctions. This vital information can reach the local people through local arrangements with the local leaders where one can give advice on the correct amount of ground material to be used in making concoctions so that local people do not consume concentrated concoctions which may be toxic. In herbal practice, *Z.chalybeum* is usually prepared as a concoction with other antimalarial plants (Nguta *et al.,* 2010), and this could explain why adverse effects have not been reported by those communities commonly using the

plant as an antimalarial phytotherapeutic remedy. Significant toxicity of *Z.chalybeum* methanolic root bark extract on human normal foetal lung fibroblast cells has also been reported by Kamuhabwa *et al.* (2000). Similarly high toxicity of stem bark of *L.cornuta* on Brine shrimp larvae has also been reported by Nguta *et al* .(2011) and together with the high toxicity of the crude leave extract of *L.cornuta* on Brine shrimp larvae witnessed in this study confirms observations from other researchers. However there are no previous reports on toxicity of *C. glaucum*.

The aim of carrying toxicity tests was to determine whether the chemosuppression observed was due to toxicity of the plant extracts or due to the ability of the plant extracts to elicit antimalarial activity. Non toxicity of *A.digitata* and *C.glaucum* extracts implies that the extracts from these plants have a potential to inhibit the growth of *Plasmodium* parasites which is not associated with toxicity. For instance, aqueous extracts of *Z.chalybeum* had a chemosuppression of 44.93% and were highly toxic (with a LD_{50} of 268.28 µg/ml) on Brine shrimp larvae implying that the toxicity of the extracts could have lead to the witnessed chemosuppression of the *Plasmodium berghei* parasites

Biological activity is attributed to the presence of various secondary metabolites in plants (Mazid *et al.*, 2011). Alkaloids and flavonoids in aqueous stem bark extracts of *A.digitata* and alkaloids, flavonoids, saponins, sesquiterpene lactones in organic root extracts of *C.glaucum* were the active constituents with antimalarial activity which reduced parasitaemia and hence prolonged the life span the mice in the experiment. Just like growth inhibition, toxicity is also attributed to presence of various secondary metabolites found in the plants extracts. Not only their presence, but also the quantity of the phytochemical constituents in a given plant extract determines the extent of extracts' bioactivity. In addition, presence of more than one class of secondary

metabolites in a given plant extract will also determine the nature and extent of extract's biological activity (Wang *et al.*, 2010)

All the plant extracts used in the experiment were screened using TLC for the presence or absence of various classes of secondary metabolites. Alkaloids and flavonoids were found to be present in all crude plant extracts. Sesquiterpene lactones were present in the organic extracts of *A.digitata, C.glaucum, L.cornuta* and *Z.chalybeum.* Saponins were present in organic extracts of *A.digitata, C.glaucum, L.cornuta and Z.chalybeum* and aqueous extracts of *L.cornuta.* Alkaloids, flavonoids and sesquitepenes have been reported to be potent plant secondary metabolites with broad spectrum of bioactivities (Mazid *et al.*, 2011). Alkaloids are major classes of compounds possessing antimalarial activity; quinine is one of the most important and oldest antimalarial drug which belongs to this class of compounds (Saxena *et al.*, 2003)

A.digitata stem bark contains medicinal compounds which are largely classified under saponins, alkaloids and flavonoids such as lupeol acetate, β-sitosterol, scopoletin, friedelin, betullinic acid and adansonin while the fruit pulp of *A.digitata* is rich in procyanidins (Sidibe & Williams, 2002; Shahat, 2006). Adansonin, an alkaloid has been proved to be one of the active principles for treatment of malaria and other fevers (Sidibe & Williams, 2002). Thirteen alkaloids have been isolated from tubers of *Stephania erecta* Will. (Menispermaceae) while two alkaloids; villastonine and alstonerine have been isolated from root extracts of *Alstonia angustifolia* Wall.ex. A. DC. (Apocynaceae). These alkaloids from *S.erecta* and *A.angustifolia* have been shown to have *in vitro* antimalarial activity against *P.falciparum* (Saxena *et al.*, 2003). In addition, alkaloids from methanolic leaf extract of *Annona senegalensis* Pers. (Annonaceae) have been reported to play a role in the growth inhibition of *Plasmodium berghei* parasites in mice (Ajaiyeoba *et al*, 2006). Flavonoids such as genistein and pratensein isolated from the leaf and

stem bark of *Andria inermis* (W.wright) DC. (Leguminosae) have *in vitro* antimalarial activity against *P.falciparum* (Saxena *et al.*, 2003). Cowanin and Cowanol are two flavonoids isolated from the ethanolic stem bark extracts of *Garcinia cowa* Roxb. (Guttiferae) which also have antiplasmodial activity against *P.falciparum* (Saxena *et al.*, 2003). Other compounds with potent antimalarial activity include various sesquiterpene lactones such as artemisinin isolated from the leaf extracts of *Artemisia annua* L. (Compositae) and arteinculton isolated from leaf extracts of *Artemisia maritima* L. as reported by Saxena *et al.* (2003). Peroxycalamenene and patchoulenone are sesquiterpene lactones isolated from tuber of *Cyperus rotundus* L. (Cyperaceae) which are active against chloroquine-resistant strains of *P.falciparum* (Saxena *et al.*, 2003). Artemisinin is a novel sesquiterpene lactone which is safe and effective antimalarial agent even in case of chloroquine-resistant strains of *P. falciparum* (Akira *et al.*, 2001). Other studies have reported that saponins isolated from methanolic and chloroformic leaf extracts of *Taminalia cattapa* L. (Compretaceae) were active against *P. falciparum* (Mudi & Mohammed, 2009). *Vernonia ambigua* Kotschy & Peyr. (Asteraceae) whole plant aqueous extract contains saponins which have *in vitro* antiplasmodial activity against *P.falciparum* as argued by Builders *et al.*, (2011). Results from phytochemical screening of the plant extracts confirm the presence of the classes of compounds mentioned above. Hence these are the compounds which could have been responsible for the reduction of growth of *Plasmodium* parasites in the mice.

The above study is useful in treating malaria infections and in malaria drug discovery because it is based on mouse models and *P.berghei*. All mammalian malaria parasites behave in a similar manner when they are in their hosts. Studies done using *P.berghei* will correlate very well with studies done using human malaria parasites like the most infectious *P.falciparum* (Sinden,1978) Aqueous extracts of *A.digitata* have been found to have *in vivo* antimalarial activity against

P.berghei from the current study. Similar studies like this are important so as to identify plants with antimalarial activity from plant diversity which can open new avenues for further studies as far malaria drug discovery and treatment of malaria is concerned such as *in vitro* studies whereby *P.falciparum* can be tested on the various plant botanicals and further phytochemical analyses to identify active constituents in the various plant botanicals. Once plants with good antimalarial activities have been identified, they can be cultivated and conserved in large scale so that they can be easily available. For instance *A.annua,* a plant with antimalarial activity which is a major source of artemisinin, one of the major components of Artemisin Combined Therapies is grown in Kenya, Madagascar, Tanzania and Uganda in large scale (Brisibe *et al.*, 2008)

6. CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

C. glaucum and *A.digitata* are two plants which from the current study have been shown to inhibit the growth of *Plasmodium berghei* parasites. Indeed, aqueous extracts of *A.digitata* had a chemosuppression of 60.47% implying that, this is a plant with good antimalarial activity against *Plasmodium berghei* parasites. The stem bark of *A.digitata* and roots of *C.glaucum* have been proved to be non toxic on Brine shrimp larvae. This leads to the conclusion that *Plasmodium berghei* growth inhibition by the two plants was not associated with their toxicity and suggests that *A.digitata* and *C.glaucum* may be potential candidates for further antimalarial drug research particularly *A.digitata* because it was found to have high growth inhibition on *Plasmodium berghei* parasites

Z.chalybeum stem back extracts and *L.cornuta* leaf extracts on the contrary were shown to be highly toxic to Brine shrimp larvae and possessed moderate antimalarial activity implying that their parasite growth inhibition could be tied to their toxicity. This study reveals for the first time *in vivo* antimalarial activity of *C.glaucum* and *L.cornuta*. In addition this study confirms the antimalarial activities of *A.digitata* and *Z.chalybeum* which have been reported before. By carrying out *in vivo* antimalarial activity, the study shows that selected four plants species used in traditional medicine for treatment of malaria have the capability of inhibiting growth of malaria parasites and consequently could have a potential in the treatment of malaria.

Flavonoids, alkaloids, saponins and sesquiterpene lactones were present in organic extracts of *A.digitata*, *C.glaucum*, *L.cornuta* and *Z.chalybeum*. Alkaloids and flavonoids were also present in aqueous extracts of *A.digitata, C.glaucum*, *L.cornuta* and *Z.chalybeum* while saponins were only present in aqueous extracts of *L.cornuta*. Further studies particularly on *in vitro* studies using *P.falciparum* and on the phytochemical analysis of *A.digitata* and *C.glaucum* are

warranted. The specific compounds present in *A.digitata* and *C.glaucum* should be isolated and investigated as potential candidates for antimalarial activity. Such studies can identify compounds present with therapeutic value in the treatment of malaria. This would help in the development of new antimalarial drugs from plant biodiversity.

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7. APPENDICES

Appendix 1: Calculation of Parasitaemia and Chemosuppression

CONTROLS

Mice		Negative Control					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	49	90	74	68	**281**	33.73	N/A
	Total Cells(TCs)	174	267	194	198	**833**		
M2	PCs	90	65	51	82	**288**	33.41	N/A
	TCs	255	204	177	226	**862**		
M3	PCs	63	73	69	69	**274**	28.42	N/A
	TCs	239	265	253	207	**964**		
M4	PCs	55	62	60	65	**242**	30.06	N/A
	TCs	227	168	203	207	**805**		
M5	PCs	75	67	72	71	**285**	33.8	N/A
	TCs	235	202	192	214	**843**		
	AVERAGE						**31.88**	**0**

Mice		Positive Control					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	9	11	12	5	**37**	4.45	86.04
	Total Cells(TCs)	210	207	216	198	**831**		
		M2						
M2	PCs	2	0	2	4	**8**	1.18	96.3
	TCs	181	170	173	155	**679**		
		M3						
M3	PCs	10	5	2	8	**25**	3.93	87.69
	TCs	166	159	142	170	**637**		
		M4						
M4	PCs	20	6	18	12	**56**		
	TCs	241	189	214	201	**845**	6.23	79.21
		M5						
M5	PCs	13	7	12	10	**42**		
	TCs	200	264	294	249	**1007**	4.17	86.92
	AVERAGE						**4.07**	**87.23**

Aqueous extracts

Mice		*Adansonia digitata* A					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	43	53	31	44	**171**	22.68	28.86
	Total Cells(TCs)	163	220	203	168	**754**		
M2	PCs	10	9	12	14	**45**	6.08	80.92
	TCs	216	214	207	103	**740**		
M3	PCs	26	14	13	18	**71**	9.05	71.63
	TCs	206	189	197	193	**785**		
	AVERAGE						**12.60**	**60.47**

Mice		*Canthium glaucum* A					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	57	77	54	98	**286**	27.5	13.74
	Total Cells(TCs)	213	251	275	301	**1040**		
M2	PCs	67	56	38	52	**213**	22.05	30.83
	TCs	212	260	227	267	**966**		
M3	PCs	66	53	55	57	**231**	26.25	17.66
	TCs	239	221	215	205	**880**		
M4	PCs	25	43	39	37	**144**	17.87	43.95
	TCs	219	202	194	191	**806**		
M5	PCs	33	36	37	39	**145**	14.75	53.73
	TCs	283	222	236	242	**983**		
	AVERAGE						**21.68**	**31.98**

Mice		*Launaea cornuta* A					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	39	28	45	39	**151**	21.7	31.95
	Total Cells(TCs)	204	163	164	165	**696**		
M2	PCs	55	63	56	49	**223**	19.65	38.37
	TCs	210	321	300	304	**1135**		
M3	PCs	30	42	30	44	**146**	16.12	49.45
	TCs	264	184	215	243	**906**		
M4	PCs	50	49	46	53	**198**	21.48	32.64
	TCs	217	242	261	202	**922**		
M5	PCs	43	37	45	41	**166**	19.69	38.23
	TCs	219	218	209	197	**843**		
	AVERAGE						**19.72**	**38.13**

Mice		*Zanthoxylum chalybeum* A					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	34	24	27	36	**121**	9.51	70.19
	Total Cells(TCs)	321	303	317	332	**1273**		
M2	PCs	48	18	37	39	**142**	12.67	60.27
	TCs	301	214	289	317	**1121**		
M3	PCs	77	56	63	58	**254**	29.16	8.53
	TCs	201	245	222	203	**871**		
M4	PCs	38	40	43	49	**170**	13.87	56.51
	TCs	308	312	297	309	**1226**		
M5	PCs	55	62	65	57	**239**	22.59	29.14
	TCs	277	261	252	268	**1058**		
	AVERAGE						**17.56**	**44.93**

$CHCl_3$: MeOH extracts

Mice		*Adansonia digitata* B					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	57	49	66	41	**213**	26.96	15.43
	Total Cells(TCs)	152	221	237	180	**790**		
		M2						
M2	PCs	80	62	60	86	**288**	24.53	23.06
	TCs	319	260	283	312	**1174**		
		M3						
M3	PCs	84	86	81	77	**328**	30.54	4.2
	TCs	292	280	269	233	**1074**		
		M4						
M4	PCs	60	64	71	70	**265**	20.75	34.91
	TCs	304	322	309	342	**1277**		
		M5						
M5	PCs	13	7	12	10	**42**	4.17	86.92
	TCs	200	264	294	249	**1007**		
	AVERAGE						**21.39**	**32.90**

Mice		*Canthium glaucum* B					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	81	50	50	39	**220**	21.63	32.15
	Total Cells(TCs)	327	233	228	229	**1017**		
		M2						
M2	PCs	50	48	35	50	**183**	22.56	29.23
	TCs	211	197	180	223	**811**		
		M3						
M3	PCs	34	31	42	38	**145**	17.22	45.98
	TCs	205	201	192	244	**842**		
		M4						
M4	PCs	23	15	14	12	**64**	8.65	72.87
	TCs	179	189	181	191	**740**		
		M5						
M5	PCs	80	62	41	29	**212**	19.58	38.58
	TCs	341	248	217	277	**1083**		
	AVERAGE						**17.39**	**43.76**

Mice		*Launaea cornuta B*					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	63	59	69	48	**239**	30.18	5.33
	Total Cells(TCs)	194	215	219	164	**792**		
		M2						
M2	PCs	6	4	3	3	**16**	1.96	93.85
	TCs	208	202	210	196	**816**		
		M3						
M3	PCs	35	57	48	38	**178**	24.83	22.11
	TCs	202	196	165	154	**717**		
		M4						
M4	PCs	34	37	49	31	**151**	22.88	28.23
	TCs	146	163	172	179	**660**		
		M5						
M5	PCs	47	59	57	46	**209**	30.07	5.68
	TCs	175	155	189	176	**695**		
	AVERAGE						**21.98**	**31.04**

Mice		*Zanthoxylum chalybeum* B					Percentage Parasitaemia	Percentage Chemosuppression
		MF1	MF2	MF2	MF2	TOTAL		
M1	Parasitized Cells(PCs)	63	65	54	78	**260**	22.69	28.83
	Total Cells(TCs)	293	256	285	312	**1146**		
		M2						
M2	PCs	70	56	75	56	**257**	20.53	35.6
	TCs	347	305	320	280	**1252**		
		M3						
M3	PCs	53	68	67	72	**260**	21.1	33.81
	TCs	321	301	278	332	**1232**		
		M4						
M4	PCs	89	79	60	77	**305**	24.19	24.12
	TCs	345	360	254	302	**1261**		
		M5						
M5	PCs	99	80	70	78	**327**	26.96	15.43
	TCs	318	279	318	298	**1213**		
	AVERAGE						**23.09**	**27.56**

Appendix 2: Multiple comparison of chemosuppressions

Multiple Comparisons

Chemosuppression(DV)

Dunnett t (2-sided)

(I) Treatments(IV)	(J) Treatments(IV)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Adansonia digitata A	Positive Control (CQ)	-26.76200	16.13576	.451	-71.9911	18.4671
Canthium glaucum A	Positive Control (CQ)	-55.25000*	13.97398	.003	-94.4196	-16.0804
Launaea cornuta A	Positive Control (CQ)	-49.10400*	13.97398	.009	-88.2736	-9.9344
Zanthoxylum chalybeum A	Positive Control (CQ)	-42.30400*	13.97398	.029	-81.4736	-3.1344
Adansonia digitata B	Positive Control (CQ)	-54.32800*	13.97398	.003	-93.4976	-15.1584
Canthium glaucum B	Positive Control (CQ)	-43.47000*	13.97398	.024	-82.6396	-4.3004
Launaea cornuta B	Positive Control (CQ)	-56.19200*	13.97398	.002	-95.3616	-17.0224
Zanthoxylum chalybeum B	Positive Control (CQ)	-59.67400*	13.97398	.001	-98.8436	-20.5044

*. The mean difference is significant at the 0.05 level.

Appendix 2 shows comparison of chemosuppression induced by each plant extract with the chemosuppression induced by the positive control (chloroquine). Significance level obtained in the pairwise comparison between chloroquine and aqueous extracts of *A.digitata* is 0.451.Which is > 0.05.Implying the chemosuppression induced by aqueous extracts of *A.digitata* and that induced by Chloroquine are not significantly different.

Appendix 3: Brine shrimp Toxicity

Aqueous extracts

	Adansonia digitata A							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
	6	4	9	1	10	0	10	0
	9	1	10	0	10	0	10	0
	8	2	10	0	10	0	10	0
Average Mortality		2.33		0.33		0		0
	Canthium glaucum A							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
	3	7	10	0	9	1	10	0
	5	5	10	0	9	1	10	0
	6	4	9	1	10	0	10	0
Average Mortality		5.33		0.33		0.66		0
	Launaea cornuta A							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
	2	8	8	2	9	1	10	0
	1	9	10	0	10	0	10	0
	3	7	8	2	10	0	10	0
Average Mortality		8		1.33		0.33		0
	Zanthoxyllum chalybeum A							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
Trial	0	10	10	0	10	0	10	0
2nd	0	10	10	0	10	0	10	0
3rd	0	10	9	1	10	0	10	0
Average Mortality		10		0.33		0		0

$CHCl_3$: MeOH extracts

	Adansonia digitata B							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
	0	10	5	5	10	0	10	0
	0	10	7	3	9	1	9	1
	0	10	8	2	9	1	10	0
Average Mortality		10		3.33		0.66		0.33
	*Canthium glaucum*B							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
	5	5	10	0	10	0	10	0
	9	1	8	2	10	0	10	0
	10	0	10	0	10	0	10	0
Average Mortality		2		0.66		0		0
	Launaea cornuta B							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
	0	10	5	5	10	0	10	0
	0	10	7	3	8	2	10	0
	0	10	6	4	9	1	10	0
Average Mortality		10		4		1		0
	Zanthoxyllum chalybeum B							
Conc.	1000µg/ml		100µg/ml		10µg/ml		0µg/ml	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
	0	10	0	10	8	2	10	0
	0	10	3	7	6	4	10	0
	0	10	0	10	10	0	10	0
Average Mortality		10		9		2		0