IN VIVO ANTIMALARIAL EFFECTS AND ACUTE TOXICITY OF SELECTED PLANT EXTRACTS USED IN MSAMBWENI DISTRICT, KENYA

BY

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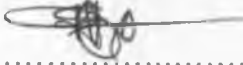
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DECLARATION

This thesis is my original work and has not been submitted for a degree in any other University.
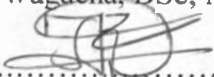
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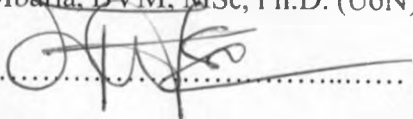
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DEDICATION

To my lovely wife Mrs. Venoricah Gicharu, dear daughter Joyjoan, parents Mr. & Mrs. James Mwangi and siblings late Virginia, Ruth, Lillian and David and late Joan.

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ABBREVIATIONS AND ACRONYMNS

ACT	Artemisinin-based combination therapy
AMFm	Affordable medicines facility-malaria
BTi	*Bacillus thuringiensis israelensis*
CAVS	College of Agriculture and Veterinary Sciences
CDC	Centre for Disease Control and Prevention
CMC	Carboxymethylcellulose
CM	Cerebral malaria
CQ	Chloroquine
DDT	Dichlorodiphyltrichloroethane
DMSO	Dimethyl sulphoxide
EPI	Expanded program for immunization
ID_{50}	Infectious dose 50
ITNs	Insecticide treated nets
IVM	Integrated vector management
KEMRI	Kenya Medical Research Institute
LC_{50}	Lethal concentration fifty
MDR	Multidrug resistance
PATH	Program for appropriate technology in health
PBK	Pyrethrum Board of Kenya
P*f*API	*Plasmodium falciparum* annual parasite incidence
PRBC	Parasitized red blood cells
RBM	Roll back malaria partnership

RD	Respiratory distress
RDT	Rapid diagnostic tests
R&D	Research and development
SMA	Severe malaria anaemia
SP	Sulphadoxine-pyrimethamine
USD	United States dollars

ABSTRACT

One of the immediate challenges that Africa is facing in the fight against malaria is spread of the parasite's drug resistance. As a result of this trend, Kenya among other endemic sub-Saharan countries would have to turn to other more expensive drugs, for example artemisinin or combinations of drugs. There is therefore an urgent need to step up research effort to develop effective drugs that are affordable to low income population. The objective of this study was to determine *in vivo* antimalarial effect on *P. berghei-* infected mice and their acute toxicity using Brine shrimp lethality test, of five selected aqueous plant extracts, *Azadirachta indica* (L), Burm (Meliaceae) root bark extract, voucher specimen number JN08; *Dichrostachys cinerea,*(L) Wight et Arn (Mimosaceae), root extract, JN16; *Grewia trichocarpa* Hochst ex A. Rich (Tiliaceae), root extract, JN022; *Tamarindus indica* L., (Caesalpiniaceae), stem bark extract, JN038; and *Acacia seyal* (Del), (Mimosaceae), root extract, JN01. These plants were collected in August 2009 from Msambweni district, Kenya based on their traditional reputation for their use as antimalarials by Msambweni community. To evaluate antimalarial effect, each plant extract was administered into chloroquinee (CQ) sensitive *Plasmodium berghei*-infected Swizz mice, after which percentage chemosuppression was determined after four days of infection. To evaluate acute toxicity of aqueous plant extracts, Brine shrimp lethality test was used where *Artemia salina* nauplii were inoculated into 3.3% solution of artificial sea water at varying concentrations of test extracts and percentage death determined after 24 hours. The lethal concentration fifty (LC_{50}), at 95% confidence interval and slope were determined from the 24 hour counts using the Finney computer programme. The five screened plant extracts suppressed parasitaemia in the following increasing order: *A. indica*, 3.1%; *D. cinerea*, 6.3%; *T. indica*, 25.1%; *A. seyal*, 27.8% and *G. trichocarpa*, 35.8% while chloroquine (positive control) had a

chemosuppression of 100%. One (20%) of the plant extracts had an LC_{50} of 285.8µg/ml, and was considered moderately cytotoxic. Two (40%) of the plant extracts had an LC_{50} of 516.4 and 545.8 µg/ml and were considered to be weakly cytotoxic while two (40%) of the crude plant extracts had acute toxicity of 1000 µg/ml and above and were therefore non-toxic. The results indicate that the aqueous extract of tested plant extracts when used alone as monotherapy, had a non-significant ($P \geq 0.05$) antimalarial activity compared to that of chloroquine (100%). The results also suggest that the anecdotal efficacy reported by the study community is related to synergism of phytoconstituents since the assayed plant parts are used in combination with others to treat malaria. It is also evident that none of the screened plant extracts is toxic to the arthropod invertebrate, *Artemia salina* L. (Artemiidae) larvae, justifying the continued use of the plant parts to treat malaria. Future work is suggested to evaluate the antimalarial activity of organic extracts of the studied plant parts.

Key words: *Plasmodium berghei, P. falciparum,* drug resistance, malaria, aqueous plant extracts, acute toxicity and albino mice.

CHAPTER ONE: INTRODUCTION

1.1 Background

Malaria is a vector-borne infectious disease caused by the protozoan Plasmodia parasites. There are four types of *Plasmodium* species that cause malaria namely *P. falciparum, P. vivax, P. malariae* and *P. ovale* while the vector carrying and transmitting the disease is of the female *Anopheles* mosquito species (Toure *et al.,* 2004). *Plasmodium* depends on two hosts, a female *Anopheles gambiae* mosquito and a human to complete its life cycle. The disease is widely spread in tropical and subtropical regions, including most of Sub-Saharan Africa, Asia and the Americas. Malaria is prevalent in these regions because of the significant amounts of rainfall and consistent high temperatures; warm and high humidity, along with stagnant waters that provide mosquitoes with the environment needed for continuous breeding (Prothero, 1999).

It is estimated that about 80 % of all malaria deaths in the world occur in sub-Saharan Africa (WHO, 2003). Majority of infections in Africa are caused by *Plasmodium falciparum,* the most lethal of the four human malaria parasites. Additionally, the most effective malaria vector *Anopheles gambiae* is the most widespread in Africa and the most difficult to control (WHO, 2002). It is estimated that one million people in Africa die from malaria each year and most of these are children under the age of 5 years (WHO, 2002). In Kenya, 22 million people are at risk of malaria, 70% of them are in rural areas. About 34,000 Kenyan children die every year from malaria compared to a total estimate of 42,000 deaths (Director of Medical Services, Kenya, 2006). Furthermore, the disease not only results in loss of life and productivity because of illness and premature deaths, it also

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hinders children in their schooling and social development both through absence from school and permanent neurological or other damage associated with severe episodes of the disease (Tabuti, 2008).

The pathophysiology of malaria has two phases: an exoerythrocytic and an erythrocytic phase. The exoerythrocytic phase involves infection of the hepatic system, or liver, whereas the erythrocytic phase involves infection of the erythrocytes, or red blood cells (Bledsoe, 2005). When an infected mosquito pierces a person's skin to take a blood meal, sporozoites in the mosquito's saliva enter the bloodstream and migrate to the liver. Within minutes of being introduced into the human host, the sporozoites infect hepatocytes, multiplying asexually and asymptomatically for a period of 8–30 days (Bledsoe, 2005). These organisms then differentiate to yield thousands of merozoites. This is followed by rupture of their host cells and release of merozoites into the blood and infects red blood cells, thus beginning the erythrocytic stage of the life cycle (Bledsoe, 2005). Malaria is managed by both chemoprophylaxis and therapy using antimalarial drugs. Control of mosquito is also important.

1.2 Problem statement and justification

Resistance to antimalarial drugs is proving to be a challenge in malaria control in most parts of the world. Since early 60's, the sensitivity of the parasites to chloroquine, the best and most widely used drug for treating malaria, has been on the decline. The loss of effectiveness of chemotherapy constitutes the greatest threat to the control of malaria. Therefore, to effectively manage malaria, new knowledge, products and tools are urgently needed and particularly new drugs (Omulokoli *et al.* 1997). New antimalarial drugs were

discovered in an effort to tackle this problem, but unfortunately the drugs are either expensive or have undesirable side effects. World Health Organization (2007) came up with a strategy of using Artemisinin-based combination therapy (ACT) as a first line of treatment of malaria. However, membrane feeding assays showed that around 60% and 40% of children treated with artemether-lumefantrine and sulphadoxine-pyrimethamine plus artesunate, respectively, are still infective to mosquitoes 14 days after the beginning of the treatment (Bousema *et al.,* 2006), indicating that gametocytaemia reduction after ACT treatment is a slow process, requiring 3-4 weeks (Mens *et al.,* 2008). This represents an obvious limitation for the potential of ACT as a transmission-blocking tool. Also, the first evidences of *P. falciparum* resistance to artemisinin derivatives (Dondorp *et al.,* 2009) are casting a shadow over the future of malaria control with ACT.

An effective malaria vaccine would be the most powerful and most cost-effective measure with a potential long-term impact even including the possibility of eradicating the disease. High levels of protection conferred through vaccination could free vulnerable population from the disease for many years, thus saving high and recurrent treatment costs. While substantial progress has been made, a real breakthrough towards a malaria vaccine is still missing.

Natural products could provide starting points in drug discovery. For decades, traditional herbal medicine has constituted a good basis for anti-malarial lead discovery and drug development. A typical example is quinine, which was the first anti-malarial drug of plant origin, isolated from the bark of *Cinchona* tree (Rubiaceae) in 1820 (Achan *et al.,* 2011).

The vastly unexplored flora and fauna (90% of total species) could provide other new leads and drugs for chemotherapy, for the isolation of certain natural products in large amounts, total synthesis by chemical approaches, or limited scope for chemical modification. The validation of traditional remedies can be problematic because of the lack of sufficient information, documentation and standardization of extracts to be evaluated, but these remedies deserve deep and thorough consideration (Corson *et al., 2007*). In spite of this problem, such materials serve as a valuable source for novel compounds. They also conceal an abundant combination of secondary metabolites which might act in synergy to enhance the therapeutic effect (Keith *et al., 2005*). Because the quantitative and qualitative composition of secondary metabolites in a plant is notoriously varied, standardization is obligatory.

The Digo community use traditional herbal remedies to treat malaria. There is need to screen traditional anti malarial herbs used by the Digo community of Msambweni district with the aim of adding to the database of scientifically approved standard plant extracts, such that from these plant extracts new anti malarial drugs might be developed. The safety of the remedies should also be tested in order to minimize toxic effects during treatment.

1.3 Hypotheses of the study

H_{O:}

The five tested plant extracts lack *in vivo* anti malarial activity and are acutely toxic to Brine shrimp larvae.

1.4 Objectives of the study

1.4.1 General objective

To determine the *in vivo* anti malarial effects and acute toxicity of selected aqueous plant extracts.

1.4.2 Specific objectives

i. To determine the *in vivo* anti malarial effect of five aqueous plant extracts using *Plasmodium berghei* – infected Swizz mice.

ii. To evaluate acute toxicity of five aqueous plant extracts using Brine shrimp lethality test.

CHAPTER TWO: LITERATURE REVIEW

Malaria is thought to have been the greatest selective pressure on the human genome in recent history (Kwiatkowski, 2005) This is due to the high levels of mortality and morbidity caused by malaria, especially the *P. falciparum* species.

2.1 Aetiology and symptoms of malaria

2.1.1 Aetiology

Malaria is a life-threatening parasitic disease transmitted by mosquitoes. It was once thought that the disease came from fetid marshes, hence the name *mal aria* (bad air). In 1880, scientists discovered the real cause of malaria: a single-cell parasite called *Plasmodium* (Anonymous).

2.1.2 Lifecycle of malaria parasite in humans

Malaria infection in the human host starts when the sporozoites are injected into the blood stream during a blood meal by an infectious mosquito. The sporozoites remain in circulation for a short period, before they actively enter the liver of the host and invade the hepatocytes and start the asexual exo-erythrocytic schizogonic cycle (Lopez-Antunano, 1980). The liver strophozoites begin to develop and multiply asexually, to form mature schizont (the multinucleated stage of the parasite) and finally a large number of merozoites are released.

The blood phase of the life-cycle is initiated when the merozoites from liver are discharged into circulation (Garnham, 1988). It invades almost immediately an erythrocyte to enter its

trophozoite stage. A vacuole is produced by the parasite which assumes the characteristic ring form (the young trophozoite). Within 12-24 hours, as the parasite grows, the cytoplasm expands, the vacuole slowly disappears and a characteristic parasitic pigment becomes visible within the cytoplasm. At the end of this phase the trophozoite has a single nucleus, a large cytoplasm, no vacuole, and a variable amount of pigment. The nucleus starts to divide approximately 30 hours after invasion in the case of *P. falciparum*, *P. vivax*, and *P. ovale*, while in *P. malariae* this requires approximately 40 hours. As nuclear division produces two or more nuclei, the parasite enters the stage of a schizont. Nuclear division continues until an appropriate number of merozoites are produced: approximately 36 for *P. falciparum*, 24 for *P. vivax* and *P. ovale*, 12 for *P. malariae*. At the end of this phase the schizogonic cycle is completed, the erythrocyte ruptures releasing the merozoites into the blood stream and determining the typical malaria paroxism.

The merozoites discharged into the circulation invade new erythrocytes to repeat the schizogonic cycle until the process is inhibited by the specific immune response or by chemotherapy. In the course of a schizogonic cycle (within a red blood cell) some of the merozoites become differentiated into sexual forms (the gametocytes); the mechanisms at the basis of this differential development are unknown. Gametocytes appears early (approximately from the third generation) in infections caused by *P. vivax*, *P. ovale*, and *P. malariae*, while at least 10 generation are thought to be required before *P. falciparum* gametocytes appears in the blood, which probably reflects the slow maturation and the sequestration of the immature stages in this species (Carter, 1980). The first stage of the maturation process is the ring form. The ring form is the only asexual stage usually

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identifiable in the peripheral blood of patients with *P. falciparum* infection. *P. falciparum* gametocytes present a typical banana like shape. Two types of gametocytes are found in the peripheral blood: the female macrogametocytes and the male micro-gametocytes (Carter, 1980).

2.1.3 Pathophysiology of malaria infection

Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia (caused by hemolysis), hemoglobinuria and retinal damage (Beare *et al.*, 2006). The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections; and every three days for *Plasmodium malariae. Plasmodium falciparum* can have recurrent fever every 36–48 hours or a less pronounced and almost continuous fever. For reasons that are poorly understood, but that may be related to high intracranial pressure, children with malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage (Idro *et al.*, 2005). Malaria has been found to cause cognitive impairments, especially in children. It causes widespread anaemia during a period of rapid brain development and also direct brain damage. This neurologic damage results from cerebral malaria to which children are more vulnerable (Boivin, 2002). Cerebral malaria is associated with retinal whitening (Maude *et al.*, 2009), which may be a useful clinical sign in distinguishing malaria from other causes of fever (Beare *et al.*, 2006).

2.2 Malaria transmission

Environmental and socio-economic changes, such as global warming, deforestation, commercial development and construction of water-control systems, are expected to exert a huge impact on the transmission of viral and parasitic diseases such as malaria (Githeko *et al.*, 2001).

2.2.1 *Anopheles* mosquito

There are approximately 460 recognized *Anopheles* species: while over 100 can transmit human malaria, only 30–40 commonly transmit parasites of the genus *Plasmodium*, which cause malaria in humans in endemic areas (Lehrer, 2010). *Anopheles gambiae* is one of the best known, because of its predominant role in the transmission of the most dangerous malaria parasite species (to humans) – *Plasmodium falciparum*. The name comes from the Greek αν, *an*, meaning *not*, and όφελος, *óphelos*, meaning *profit*, and translates to *useless* (Steven, 2010).

2.2.2 Conditions necessary for breeding of *Anopheles* mosquito

Anopheles gambiae sensu stricto (Diptera: Culicidae), the major African malaria vector is known to breed in temporary clean and clear water (Service, 1971). But, the rapid unplanned urbanization observed in many parts of Africa is changing the context of human population and natural ecosystem interaction. Poverty, deteriorating infrastructure and overcrowding are some of the factors that contribute to the development of conditions that modify anopheline breeding sites.

2.2.3 Life cycle of malaria parasite in *Anopheles* mosquito

The sporogonic cycle initiates when mature female and male gametocytes are ingested by a suitable species of *Anopheles* during a blood meal. As soon as gametocytes reach the midgut of the insect the female gametocyte shed the red blood cells and remains free in the extracellular space as a macrogamete. The male gametocyte nucleus divides into eight spermlike flagellated microgametes each of which also leaves the erythrocyte, reaches the midgut and actively moves to fertilize a macrogamete. The result of the fertilization process is the zygote, which develops into the elongated, slowly motile ookinete within 18 hours from the blood meal. The ookinete actively penetrates the peritrophic membrane and the epithelium of the midgut and settles beneath the basal lamina of the outer gut wall, where it develops into a non motile oocyst. The oocyst nucleus divides repeatedly leading to the formation of as many as 10,000 new individual nuclei within a mature oocyst. The product of the mature oocyst is the sporozoites, narrow and curved in shape that is actively motile. The sporozoites actively leave the cyst and reach the salivary glands and finally settle into the salivary duct. When the mosquito feeds, the salivary fluid (which has anti-clotting properties) and its content of sporozoites are actively injected into the vertebrate host to start another asexual replicative cycle.

2.3 Factors affecting malaria transmission

Several factors are found to be associated with the occurrence and abundance of anopheline larvae that could be classified into the following four categories: climatic factors (rainy season), environmental factors (lower level of urbanization, higher water

temperature, higher pH) and biological factors (increased surface vegetation, and the absence of larvivorous fishes).

2.3.1 Infectivity of human carriers

In regions with seasonal transmission characterized by a long dry season, strains of *P. falciparum* with a long duration of infectivity present a selective advantage on a strain with a short one which is unlikely to be transmitted from one transmission season to the other. In regions with continuous transmission, this selective process will not be required (Carnevale and Mouchet, 1980). On the other hand the adaptation of parasitological cycle to different local climatic conditions has well been documented for *P. vivax* (Bray and Garnham, 1982).

2.3.2 Vector density

The vector density in relation to humans will be dependent on the type of the breeding place and the distance to the human habitat. Nature of breeding places of *Anopheles gambiae* complex, shallow open sun-lit pools assumes a large distribution of these species in tropical Africa. Vector density and thus malaria increases with clearing of forests (Mouchet, 1976).

2.3.3 Environmental factors

The development of the parasite within the mosquito (sporogonic cycle) is dependent on temperature. It takes about 9 to 10 days at temperatures of 28 °C, but stops at temperatures

below 16 °C. The minimum temperature for parasite development of *P. falciparum* and *P. vivax* is estimated to be 18 °C and 15 °C, respectively (Craig *et al.,* 1999).

The level of floating vegetation is positively associated with the presence of larvae. This could be explained by the difficulties of measuring the surface of vegetation that covers a water collection. Also, the surface vegetation can be a proxy for the presence of food, which can favour the presence of anopheline larvae.

The pH is significantly associated with the occurrence or the abundance of larvae, and this factor could also be considered to be an indicator of the presence of food for larvae. The presence of predator fishes is associated with a lower probability of larvae or lower larval density (Craig *et al.,* 1999).

Numerous studies have been conducted on the impact of urban agriculture on malaria transmission (Klinkenberg *et al.,* 2008). Most specifically, urban agricultural activities can provide breeding and resting sites for malaria vectors. Market-gardens provide resting sites for adult *Anopheles*, rather than increase the number of larval habitats, as was previously demonstrated in Ghana (Klinkenberg *et al.,* 2008). Indeed, the water collections that are located in market-gardens less frequently harbour anopheline larvae, and when anopheline larvae are present, the densities are lower than in the surrounding areas. However, one cannot exclude that this effect occur because of the use of pesticides by urban farmers (Tia *et al.,* 2006).

2.4 Epidemiology

The global spatial limits of *P. falciparum* malaria transmission have been mapped (Guerra *et al., 2007*). The results of this exercise stratify the world into three classes: the spatial representation of no risk, unstable risk (*P. falciparum* annual parasite incidence [*Pf*API] < 0.1 per 1,000 people per annum [pa]), and stable risk (*Pf*API ≥ 0.1 per 1,000 people pa) of *P. falciparum* transmission for 2007 (Figure 1). Eighty percent of Africa's population was estimated to reside in an area with parasite prevalence in 2 to 10-year-olds of >5 % and 50% in an area with prevalence > 40 % (Hay *et al.,* 2008).

Stable areas (*Pf*API ≥ 0.1 per 1,000 p.a)
Unstable areas (*Pf*API < 0.1 per 1,000 p.a)
No risk areas (*Pf*API = 0 per 1,000 p.a)
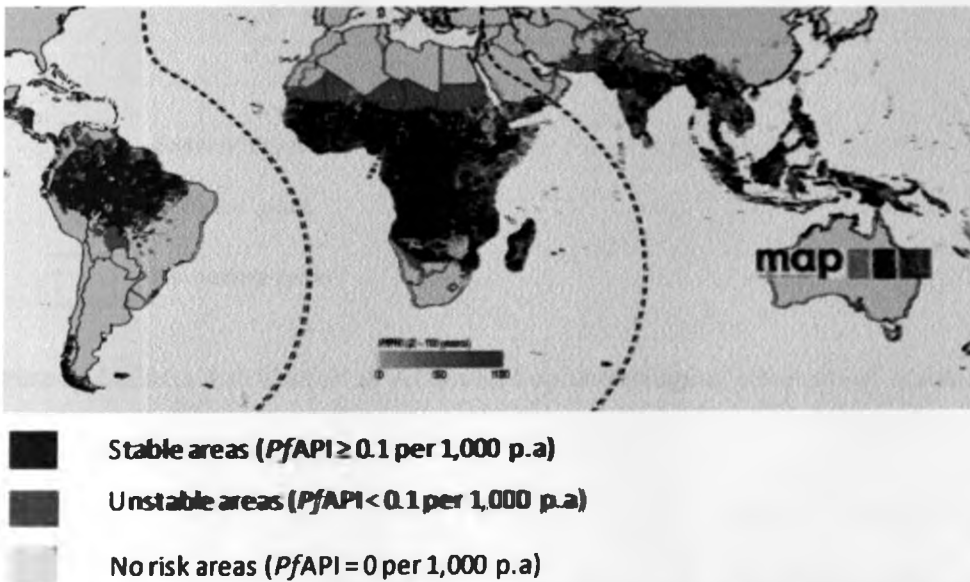
Figure 1: Map showing global malaria distribution. (Map courtesy of Hay *et al.,* 2007)

In Kenya, distribution of malaria is not uniform, because of geographical differences in altitude, rainfall and humidity. These factors influence transmission patterns, as they determine vector densities and intensity of biting. The country may be divided into three malaria ecozones: 1) stable malaria (Nyanza, Coast, and Western provinces, 2) highlands

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prone to malaria epidemics (mainly in Rift Valley Province and some parts of Nyanza Province), and 3) malaria free (Nairobi and some parts of Central Province) (Figure 2).

Endemic areas

Epidemic areas

No malaria areas
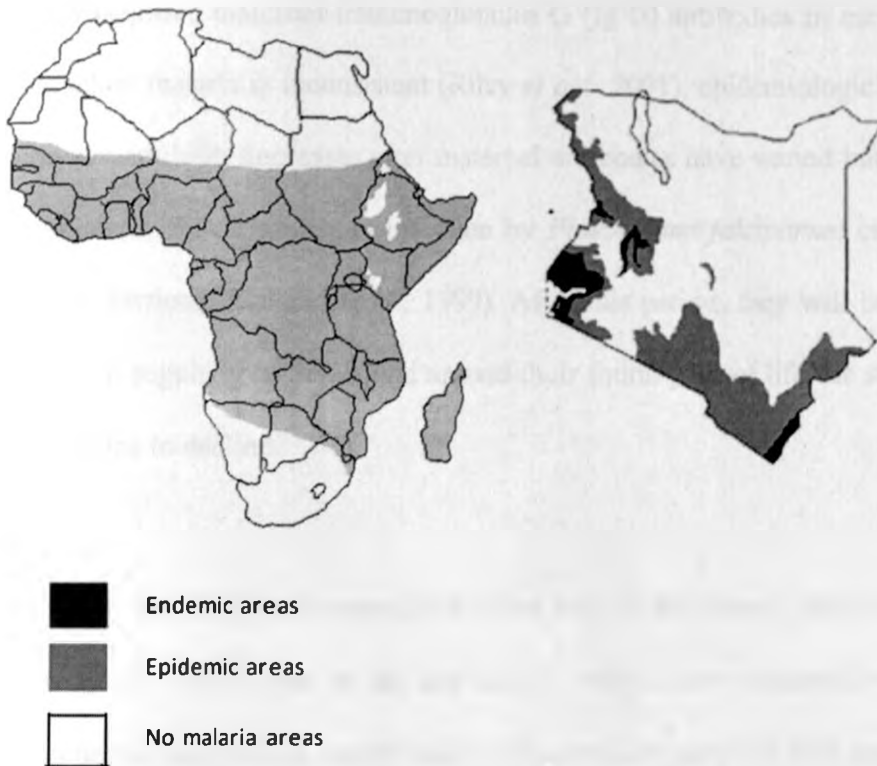
Figure 2: Malaria distribution in Africa and epidemiological situation of malaria in Kenya
Source: MARA/ARMA, 2002.

Not everybody who gets an infection becomes seriously ill or dies, because this depends on the degree of immunity of the individual. A substantial level of immunity to malaria may already be acquired after one or two infective bites. The antigenic variation of *P. falciparum* (i.e. the variation in antigens expressed at the surface of the infected red blood cell) is thought to play an important role in this (Day and Marsh 1991; Gupta *et al.*, 1999; Roberts *et al.*, 1993).

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During their first 3 to 6 months of life, infants have a protective immunity through antibodies they obtained from their mother. Although evidence supporting a direct role for passively acquired maternal Immunoglobulin G (Ig G) antibodies in mediating protection against infant malaria is inconsistent (Riley *et al.*, 2001), epidemiologic studies show that malaria susceptibility increases after maternal antibodies have waned but before immunity to blood-stage. Susceptibility to infection by *Plasmodium falciparum* can develop due to repeated infections (Bloland *et al.*, 1999). After this period, they will build up protective immunity, if regularly infected, and around their fourth year of life the severity of malaria attacks begins to decline.

Topography has long been recognized to be one of the factors associated with malaria (Cohen *et al.*, 2010) due to its association with cooler temperatures that slow the development of anopheline vectors and the *Plasmodium* parasites they transmit (Minakawa *et al.*, 2006). Unlike in lowland plains, where drainage is poor and mosquito breeding habitats have an extensive distribution, the majority of breeding habitats in the hilly highlands are confined to the valley bottoms because the hillside gradients provide efficient drainage (Minakawa *et al.*, 2005). Variation in the local topography of the land may also play an important role in determining regions of suitability for mosquito breeding at smaller spatial scales (Balls *et al.*, 2004). Depending on the variation in local topography, malaria risk may diminish within a few hundred meters from known breeding sites (Gunawardena *et al.*, 1998), although a number of vector and environmental factors have been found to influence this range (Ribeiro *et al.*, 1996).

2.5 Treatment and prophylaxis

Treatment of malaria depends on: the species of the infecting parasite and the density of that parasite in the bloodstream. Other relevant factors for treatment include the parasite's drug resistance status and the country where the parasite was contracted; any accompanying illnesses and drug allergies.

The World Health Organization (2000) suggests that treatment begin within 24 hours after symptoms appear, particularly with a *Plasmodium falciparum* infection because of its rapid progression towards severe malaria. If the species of parasite cannot be immediately identified, the patient should be treated as if infected with *P. falciparum* until the infecting species can be identified. The drug(s) administered depends on the identified parasite species and drug resistance in the region where the parasite was acquired. Travel history is especially crucial in the identification and drug resistance process. Anti-malaria drugs can be given orally, intravenously, or as a suppository determined by the severity of the infection. Most drugs are given when the parasite has a high density in the bloodstream. For example, patients who have *P. falciparum* are given continuous intravenous (IV) infusions because oral medication will not be effective.

People living with HIV and AIDS are especially vulnerable to malaria and will suffer more often and more severely from malaria once their immune system starts to decline. HIV not only increases the incidence and severity of malaria, it also compromises malaria treatment. HIV infection can decrease the response to standard anti malarial treatment. For

HIV positive adults with a weakened immune system (a low CD4 count), anti malarial drugs are less likely to be effective (The Link between Malaria and HIV, 2006).

Over-diagnosis of malaria may be widespread in sub-Saharan Africa and may lead to under-treatment for other life-threatening conditions such as septicemia. Even where resources are extremely limited, investigation for malaria especially among febrile neonates not responding to standard antibiotics treatment should be undertaken. However, in many instances, a positive slide may not explain the cause of illness (Mwaniki *et al.*, 2010).

There is now substantial evidence that using a combination of drugs with independent modes of action and different biochemical targets is not only more effective, but also successful in preventing or slowing the development of resistance, because the probability of parasites being simultaneously resistant to two drugs is greatly reduced. The rationale for combining antimalarials with different modes of action is twofold: (1) the combination is often more effective; and (2) in the rare event that a mutant parasite that is resistant to one of the drugs arises de novo during the course of the infection, the parasite will be killed by the other drug (WHO, 2006).

This thinking has been applied for some time to the treatment of tuberculosis and leprosy and, more recently, to HIV/AIDS (De Cock, 1998). In malaria treatment, using the combination drug approach with artemisinins means using artemisinin-based combination therapy, or ACT. Studies conducted in Africa have shown that, when artesunate was

added to Sulphadoxine-Pyrimethamine (SP) or amodiaquine treatment, parasite loads and gametocyte rates declined significantly faster (Olliaro, 2001).

One of the global initiatives currently underway is the Affordable Medicines Facility-malaria (AMFm), which aims to expand access to affordable ACT. The AMFm seeks to reduce consumer prices through price negotiations and a buyer co-payment for which both public and private first-line buyers at the country level are eligible. Reduced prices are expected to extend down the antimalarial supply chain so that when children's caregivers seek fever treatment at a given outlet, they are more likely to find effective medicines that they can afford.

More information on consumer and provider practices could guide interventions to address both public and private sector deficiencies. Where interventions are developed and implemented, the need for evaluation is great. Additionally, as interventions aiming to increase access to ACT are scaled up, equitable access to treatment monitored through household surveys is an important indicator of success to treatment and prevention of malaria (Sabot *et al.*, 2009).

2.6 Malaria prevention

In 1958 the World Health Organization (WHO) decided to wage a global campaign against malaria. Several notable attempts are being made to eliminate the parasite from sections of the world or to eradicate it worldwide. In 2006, the organization 'Malaria No More' set a public goal of eliminating malaria from Africa by 2015, and the organization plans to

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dissolve if that goal is accomplished (Strom, S., 2011). The interventions employed have included:

2.6.1 Use of pyrethrum or pyrethroids

Pyrethrins are currently rated as the safest alternatives to DDT because they are biological products. Toxicity of natural pyrethrum as a plant has not been established but studies on the toxicity levels of various pyrethrum products is still ongoing at the Pyrethrum Board of Kenya (PBK, 2007). The Pyrethrum Board of Kenya has recently produced pylarvex, pymos and pynet to be used in different settings in the control of malaria vector. These products (Pymos™ 0.6 EC, Pynet ™ 5 EC and 0.5 EC) consist of natural pyrethrins, synergists and emulsifiers. Under limited laboratory testing, Pymos™ 0.6 was found to have an enhanced residual capacity greater than five months.

2.6.2 Insecticide treated nets (ITNs)

Insecticide-Treated Nets (ITNs) provide protection against adult mosquitoes. The netting material is treated with synthetic pyrethroids, which is relatively safe. The insecticide repels mosquitoes and inhibits them from enjoying their blood meal even when there are large holes in the nets. The most commonly used pyrethroids are permethrin, deltamethrin and lambda cyhalothrin. Studies in Kenya, Ghana, Gambia and Tanzania found that ITNs reduced child illness by 29% to 63% and childhood mortality by between 17% to 63% depending on net coverage and malaria transmission pressure (Kenneth, 1995). These results are comparable to those of DDT in door house spraying. The main handicap of this

program is the possibilities of resistance to pyrethroids which are the only chemicals available. Also there is not yet any data on the long-term effects of ITNs.

2.6.3 Biological control

There are several types of biological control including the direct introduction of parasites, pathogens and predators to target mosquitoes. Effective biocontrol agents include predatory fish that feed on mosquito larvae such as mosquitofish (*Gambusia affinis*) and some cyprinids (carps and minnows) and killifish. Tilapia will also consume mosquito larvae (MacKay, 1995).

Other predators include dragonfly naiads, which consume mosquito larvae in the breeding waters, and adult dragonflies, which eat adult mosquitoes. Some other biocontrol agents that have had lesser degrees of success include the predator mosquito *Toxorhynchites* and predator crustaceans-Mesocyclops copepods (Marten *et al.*, 2007).

Like all animals, mosquitoes have their own set of diseases. Invertebrate pathologists study these diseases in the hope that some of them can be utilized for mosquito management. Microbial pathogens of mosquitoes include viruses, bacteria, fungi, protozoa, nematodes, and microsproidia (Davidson, 1981).

Also used as biological control agent are the dead spores of varieties of the natural soil bacterium *Bacillus thuringiensis*, especially *Bt. israelensis* (BTI). *Bacillus thuringiensis israeliasis* BTI is used to interfere in the digestive system

of larvae. It can be dispersed by hand or dropped by helicopter in large areas. However, BTI is no longer effective after the larvae turn into pupae, because they stop eating.

2.6.4 Use of vaccine

The active substance in candidate in the current malaria vaccine development is a recombinant antigen expressed in *Saccharomyces cerevisiae* coded RTS, S whose antigen consists of two proteins, RTS and S. In October 2011, preliminary findings from the Phase III trials of an experimental malaria of this vaccine reported that it could protect approximately 50% of inoculated infants and children. The RTS, S vaccine was engineered using genes from the outer protein of *Plasmodium falciparum* malaria parasite and a portion of a hepatitis B virus and a chemical adjuvant to boost the immune system response (Agnandji *et al.*, 2011).

2.6.5 Integrated Vector Management (IVM)

World Health Organisation (WHO) recommends the use of appropriate combinations of non-chemical and chemical methods of malaria vector control in the context of integrated vector management (IVM) (WHO, 2004). An IVM approach is pragmatic in that it offers a menu of vector control methods which can be applied in various combinations to suit different ecological and socioeconomic settings. Besides, by using a range of different methods, it is possible to effectively target vectors at different stages in their life cycle, for instance, as larvae and pupae in mosquito breeding habitats, or at certain times during the host-seeking and resting behaviour of adult mosquitoes (Townson *et al.*, 2005). On the other hand, reliance on only one vector control method is, in the long term, usually unsustainable for a variety of reasons, most notably insecticide resistance and adverse health and environmental impacts in the case of the use of chemical control (Rozendaal, 1997).

2.7 Antimalarial drug resistance

The World Health Organization defines antimalarial drug resistance as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within tolerance of the subject". This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bruce *et al.,* 1986; Kenya Ministry of Health, 2001).

2.7.1 Chloroquine resistance

Chloroquine resistance was first detected in Kenya in 1978, when it was still an inexpensive and widely used drug and was the medicine of choice for treating the disease. Despite reports of resistance to this drug, chloroquine was still widely used until 1998, but was eventually replaced with sulphadoxine-pyrimethamine (SP) as the first line treatment (Shretta *et al.,* 2000). The 20-year lag between initial detection of resistance and eventual policy change highlights the need for a more efficient process for detecting and responding to resistance, and guiding the course of action.

Two of the biggest contributors to antimalarial drug resistance in Kenya are self-medication and over diagnosis (Hess *et al.,* 1996). Because the symptoms of malaria overlap with those of other diseases, people who have a fever in areas with a high risk of malaria are typically prescribed antimalarial medications before a proper diagnosis can be

obtained in a laboratory. Furthermore, diagnosis for malaria is often symptomatic in a clinic, rather than a definitive diagnosis validated in a laboratory. The lack of specific and definitive malaria diagnoses may lead to over diagnosis, even in cases where the ailment is not actually malaria.

2.7.2 Sulphadoxine-Pyrimethamine (SP) resistance

In Africa, SP resistance was detected in the late 1980s, which has since spread more in the east than in the west. Kenya adopted the artemisinin-based combination treatment (ACT) artemether lumefantrine(AL) as the first-line treatment for uncomplicated malaria following the precipitous decline in the efficacy of sulphadoxine pyrimethamine (SP). Artemether lumefantrine was rolled out in 2006 with a baseline efficacy of 96 per cent. In 2008 efficacy was still 96% (Ministry of Public Health and Sanitation, 2009). Monitoring is ongoing.

2.7.3 Artemisinin resistance

Artemisinin and its derivatives are the newest and most effective anti malarial drugs. These drugs affect the protein synthesis of the parasite. In 2002–2003, decreased *in vitro* sensitivity of *P. falciparum* to Artemether was reported in French Guiana (Jambou *et al.*, 2005). Strong evidence shows that resistance to artemisinins may depend on SNPs in the drug's putative chemotherapeutic target, the SERCA-type ATPase protein (PfATP6) but epidemiological evaluation of gene copy numbers in natural parasite populations has not been carried out (Eckstein-Ludwig *et al.*, 2003).

In Kenya today, AL is offered free of charge to patients at public and mission facilities. On the market, the price of purchasing AL has dropped 93%, but it is still one of the more expensive options for treating malaria (Mwai, 2010). To date no relevant clinical resistance to artemisinins has been reported in Kenya. However, the long-term usefulness of ACT in endemic areas remains unclear (Duffy and Sibley, 2005). Therefore, as ACT becomes widely used in sub-Saharan Africa, regular and comprehensive surveillance of resistance is of great importance.

2.8 Use of herbal medicine in treatment of malaria

Herbal products are defined as herbal materials that are administered to patients and are mixtures of herbal substances and other constituents (TDR, 2005). The importance of herbal medicine practices is indicated by the fact that about 80% of the developing world's population depends on traditional medicine for their primary healthcare (Mosihuzzaman *et al., 2008*). The scientific evaluation of safety and efficacy of herbal products and medicinal preparation is thus of vital importance from both medicinal and economic perspectives.

Traditional medicine is a vital yet often neglected part of health care in Kenya. The conventional system provides for only 30% of the population, implying that more than two-thirds of Kenyans depend on traditional medicine for their primary health care needs (NCAPD, 2007). Traditional methods of malaria treatment could be a promising source of new anti malarial compounds. In Africa, more than 80% of people use traditional medicines and most families have recourse to this medicine based on plants extracts for the curative treatment of malaria (Wright and Phillipson, 1990). In fact, the traditional

medicine of this continent constitutes an important source for ethno pharmacological investigation.

Plants have been used as a source of medicine throughout history and continue to serve as the basis for many pharmaceuticals used today. The medicinal properties of plants are described already on Assyrian clay tablets dated about 2000 B.C. and documented in the Egyptian culture, the Indian Ayurveda (Patwardhan *et al.*, 2005), in Traditional Chinese Medicine (TCM) (Kong *et al.*, 2009), and various European documents. Traditional medicines not only provide valuable clues for finding new drugs, but may also help to shift the drug discovery paradigm from 'finding new-entity drugs' to 'combining existing agents', and might even direct the combinations between such agents (Kong *et al.*, 2009; Wagner *et al.*, 2009). A recent structural comparison between ~10,000 traditional TCM components and ~8,000 modern drugs or candidates, identified 908 agent pairs that are structurally similar (with similarity 0.85) and 327 agent pairs that are identical in structure. Plants continue to serve as the basis for many pharmaceuticals used today (Newman *et al.*, 2007). Although the modern pharmaceutical industry was born from botanical medicine, synthetic approaches to drug discovery have more recently become standard. Although a comprehensive review of human drugs introduced between 1981 and 2006 indicates that about 62% of new small-molecule drugs were either natural products, derived from natural products (usually semi-synthetically) and natural product–inspired pharmacophores (that could be considered natural-product analogs) (Newman *et al.*, 2007), the synthetic combinatorial chemistry and high throughput screening (HTS) of potential drug targets disconnected the historical link between plants and medicines.

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According to the International Federation of Pharmaceutical Manufacturers and Associations, the process of pharmaceutical research and development (R&D) is a complex, costly, risky and long undertaking. It requires a sustained mobilization of substantial human and financial resources over long period of time before a new drug finally reaches the patient. On average, this process takes between 10–15 years and the estimated average cost of developing a new medicine exceeds $800 million. In the course of the R&D process, more than 8,000 compounds are tested on average, of which only one is developed into a potent and safe drug (IFPMA, 2006). As a result, Pharmaceutical R & D is largely dominated by private multinational companies known to possess the financial capacities, expertise, know-how and technical excellence that guarantee the sustainability of the whole process (IFPMA, 2006).

2.9 Constraints and challenges

Herbal medicines are generally regarded as safe based on their long-standing use in various cultures. However, some of the plants used in herbal medicines can also be highly toxic (Mosihuzzaman *et al.*, 2008). As a whole, herbal medicines can have a risk of adverse effects and drug-food interactions if not properly assessed. Assessment of the safety of herbal products, therefore, is the first priority in herbal research.

Likewise, although traditional medicine is widely used to treat malaria, and is often more available and affordable than conventional medicine, it is not without limitations. Firstly, there are few clinical data on safety and efficacy. Secondly, there is no consensus, even among traditional healers, on which plants, preparations, and dosages are the most

effective. Thirdly, the concentration of active ingredients in a plant species varies considerably, depending on several factors.

Growing use of herbal medicines and the expansion of their market pose challenges in the quality and efficacy of traditional remedies and practitioners. Historically, the use of traditional medicine has been on a small local scale through provision by traditional healers often without government involvement at all. In modern times, there has been increased recognition of traditional medicine by the scientific community, media, and development plans and policies. For example, Kenya's 1989–1993 development plan made a commitment to the promotion of the welfare of traditional practitioners (Mukiama, 2005). Urban markets, on the other hand, have many herb sellers, each giving advice and selling both raw plant material and preparations that they have produced themselves. Their products are mainly packaged in bottles or small plastic bags, perhaps wrapped in newspapers, but have no indication of the appropriate dosage. Quality control is a challenge under these circumstances. So is the conservation of the botanical resource.

Both the raw herb and the extract contain complicated mixtures of organic chemicals, which may include fatty acids, sterols, alkaloids, flavonoids, glycosides, saponins, tannins, lignin, and terpenes as well as other small molecules such as peptides and oligosaccharides. It is often difficult to determine which component, if any, of the herb has biological activity in humans. In addition, the processing of herbs, such as heating or boiling, may alter the dissolution rate, or even the pharmacological activity of the organic constituents.

A major difficulty in finding single-component new-entity drugs from natural medicines originates from the fact that the efficacy of most natural medicines may lie in the synergy or additivity of diverse components rather than arising from a single compound (Houghton *et al.*, 2007; Williamson *et al.*, 2001). The synergy of natural medicines arises from the co-evolution between plants and their foes (Ma *et al.*, 2009). For example, plants developed antimicrobials to fight against the invasion of diverse pathogens. To survive the antibiotic assault, pathogens evolved resistant systems, such as multidrug resistance (MDR) pumps. In the return, plants were stimulated to develop MDR inhibitors (Tegos *et al.*, 2002). Thus, a crude extract can contain both active inhibitors and their potentiators (Jia *et al.*, 2009). Additive and synergistic effects are subsets of the pharmacodynamics of potentiation, where different compounds in a mixture interact to provide a combined effect that is equal to the sum of the effects of the individual components (additive), or where combinations of bioactive substances exert effects that are greater than the sum of individual components (synergistic).

Distinguished researchers in the field of ethnopharmacology have recommended that for the developing countries, the approval as drugs of standardized and formulated plant extracts might be the starting point of an innovative and successful local pharmaceutical industry not only for the treatment of minor diseases, but also for severe and life-threatening diseases (Pieters and Vlietinck, 2005).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

Plants used in the study were sourced from Msambweni district of Kwale County, Kenya .Msambweni district has an area of 8960km^2 It borders Taita Taveta to the west, Kilifi district to the north west, Mombasa and Indian Ocean to the east and Republic of Tanzania to the south (Figure 3). (http://www.aridland.go.ke/semi_profiles/kwale_profile.pdf (Accessed on 11th October, 2011).
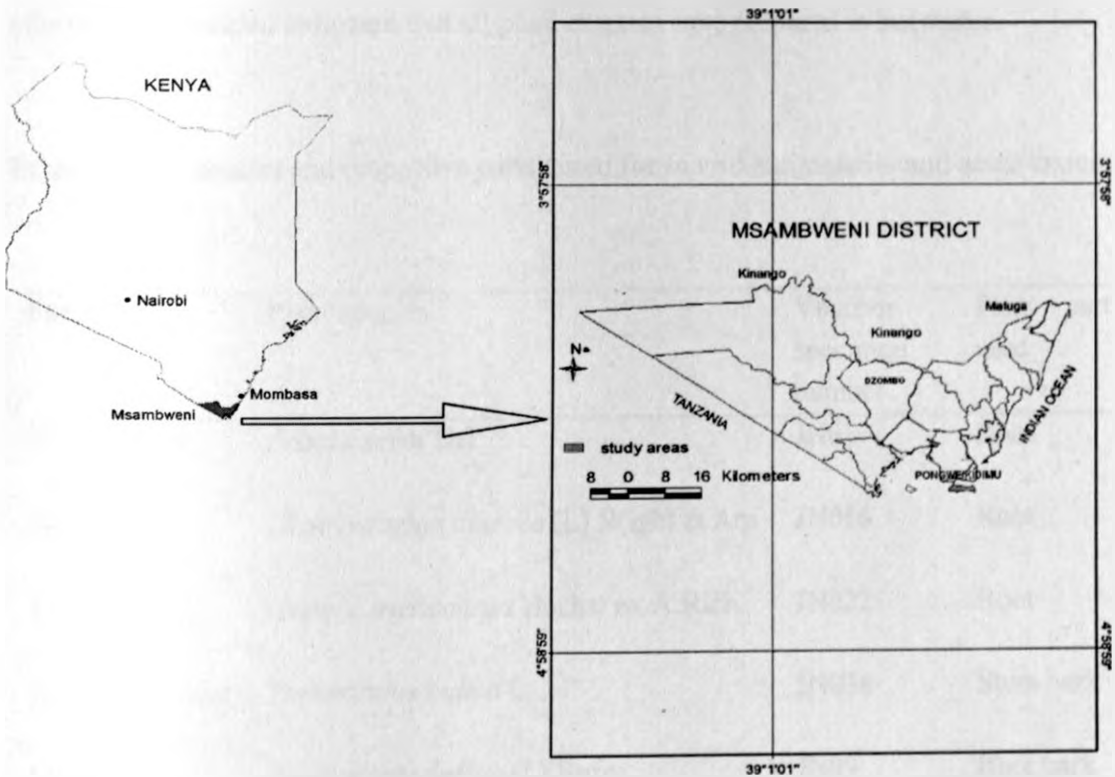
Figure 3: Map of Kenya showing Msambweni district and divisional administrative units.

(Map courtesy of Nguta *et al.*, 2010).

3.2 Collection and identification of plant materials

The plant samples used in the current study were collected in August 2009 from Msambweni district of Kenya in a previous study (Nguta *et al.*, 2010) based on ethno pharmacological use through interviews with local communities and traditional health practitioners. Permission for a sustainable plant harvesting was granted by Kenya Wildlife Service (KWS) in the forest game reserve, and the local community outside the forest areas. The information gathered included the part of the plant used (Table 1). The information provided indicated that all plant extracts were prepared in hot water.

Table 1: Plant species and respective parts tested for *in vivo* antimalarial and acute toxicity.

Family	Plant species	Voucher specimen number	Plant part used
Mimosaceae	*Acacia seyal* Del.	JN01	Root
Mimosaceae	*Dichrostachys cinerea* (L) Wight et Arn	JN016	Root
Tiliaceae	*Grewia trichocarpa* Hochst ex A.Rich.	JN022	Root
Caesalpiniaceae	*Tamarindus indica* L.	JN038	Stem bark
Meliaceae	*Azadirachta indica* (L) Burm	JN09	Root bark

The plants were identified by Mr. Kimeu Musembi, a taxonomist at the University of Nairobi Herbarium, Nairobi, where voucher specimens were deposited (Figure 4).
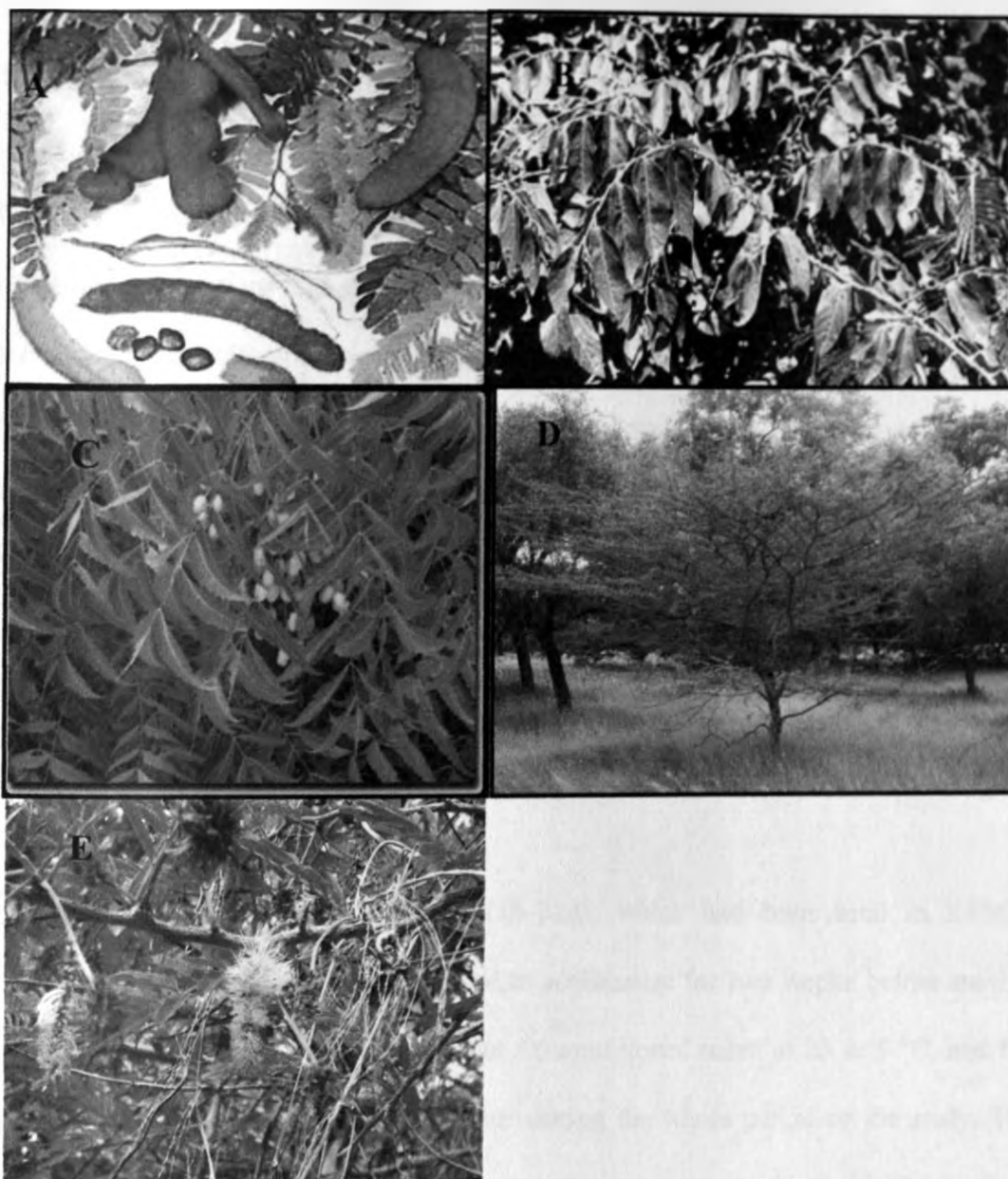
Figure 4: Photographs of plant species collected from Msambweni district, Kenya,   (A) *Tamarindus indica* L. (Tamarind tree), (B) *Grewia trichocarpa* Hochst. ex A. Rich. Burret (1910) (Mkole-Swahili), (C) *Azadirachta indica* A. Juss. (Mkilifi - Swahili, neem tree-English), (D) *Acacia seyal* Del. (red acacia - English; Mgunga- Swahili), (E) *Dichrostachys cinerea* (L.) Wight & Arn. (Sickle bush-English).

3.3 Preparation of plant extracts

The plant parts were chopped into small pieces; air dried at room temperature (25 ± 5^0C) under shade and pulverized using a laboratory mill (Christy & Norris Ltd., England). Considering that people in Msambweni usually use hot water to prepare their herbal remedies as decoctions and sometimes concoctions, aqueous hot infusions of each plant part was prepared (50 g of powdered material in 500 ml of distilled water) in a water bath at 60^0C for 1 hour. The extracts that were obtained were filtered through muslin gauze and the filtrate kept in a deep freezer for 24 hours, which was then lyophilized. The lyophilized dry powder was collected in stoppered sample vials, weighed and kept at -20^0C until used.

3.4 Determination of *in vivo* antimalarial effect of the plant extracts

3.4.1 Experimental animals

Male and female Swizz albino mice (18–22g), which had been bred in KEMRI laboratory, were used. They were allowed to acclimatize for two weeks before starting the experiment. They had been kept in an air-conditioned room at 25 ± 5 ^0C, and fed *ad libitum* with commercial food and water during the whole period of the study. The investigation and handling of animals was done in accordance to the Guide for the Care and Use of Laboratory Animals.

3.4.2 Inoculum

In vivo anti malarial testing in mice was done using chloroquine (CQ) sensitive strain of *Plasmodium berghei*. Four donor mice were injected with a CQ sensitive strain of

Plasmodium berghei, and parasitaemia allowed to build up for three days, at which point blood smears were taken on the third day, and the mice only used after ensuring that 30–40% parasitaemia was attained. After anaesthesia with chloroform, whole blood (10 ml) of donor mouse was drawn from the heart with heparinized syringe, transferred into a screw capped sterile plastic tube and then diluted to 1% using distilled water. Each test mouse was injected intra-peritoneally (ip) with 0.2 ml of the suspension (10^7 parasitized red blood cells).

3.4.3 Four day suppression test

Peters' 4-day suppressive test against *P. berghei* infection was used (Peters *et al*, 1975). After 3-hrs of infection with parasites, thirty five mice were randomly sorted by sex and assigned into seven treatment groups with each group comprising of five mice. Each group was kept in a separate cage. One group (negative control) was given 10 ml/kg body weight distilled water, one group (positive control) was given chloroquine (10 mg/kg body weight) and the remaining five groups were given 10 mg/kg body weight of five different aqueous plant extracts. Plant extracts were administered orally once daily for four consecutive days (day 0 to day 3).

3.4.4 Preparation of blood smears

On day 4 post infection, Blood was obtained from the mice for observation through tail bleeding. The first blood droplet was wiped off using clean cotton wool so as to give an accurate result. The subsequent blood was allowed to drop onto a clean microscope slide; a thin film of blood smear was made, and then fixed with methanol. The slide was stained

with Giemsa's stain for 10 min. It was then rinsed and allowed to dry in the open air and was thus ready to be viewed under the microscope with proper labeling on the various slides.

3.4.5 Counting of parasitized erythrocytes and determination of parasitaemia and chemosuppression

The stained blood smear was mounted on a microscope using immersion oil. High objective lens was used to view the slide at a magnification of ×100. The total number of parasitized erythrocytes was carefully counted as were the total number of erythrocytes. The percentage parasitaemia was obtained by the mathematical formula:

$$\% \ Parasitaemia = \frac{Total \ number \ of \ infected \ cells}{Total \ number \ of \ cells} \ x \ 100$$

Average percentage parasitaemia was taken from four fields counted per slide.

Percentage suppression of parasitaemia (chemosuppression) was calculated using the formula:

$$\% \ Chemosuppression = \frac{(Parasitaemia \ in \ negative \ control) - (parasitaemia \ in \ test \ group)}{parasitaemia \ in \ negative \ control} \ x \ 100$$

3.5 Brine Shrimp lethality test

3.5.1 Product identification, *Artemia salina* Leach (Artemiidae)

Artemia eggs, Sera premium marine salt batch number 0725701, were purchased from JBL GmbH & Co.KG (Neuhofen, Germany). The product had label JBL Artemio Pur[®] Brand.

3.5.2 Preparation of test extracts

A stock solution of aqueous extracts (10,000 μg/ml), 3.3% was prepared as follows: 33g of marine salt into 1 litre of de-ionized water and pH adjusted to 7. Dissolution was enhanced using a sonicator and the solution filter sterilized using 0.22 μm membrane filters in a laminar flow hood. Then 50 mg of test plant extract was dissolved into 5 ml of 3.3% saline solution in a 5 ml volumetric flask. Using a micro-pipette, 5 μl, 50 μl and 500 μl of test plant extract was put into separate vials. Five replicates were made for each serial dilution. The procedure was repeated for the other four plant extracts.

3.5.3 Culture and harvesting of brine shrimp, *Artemia salina*

Artemia salina eggs were stored at -20 ^{0}C before use. The eggs were incubated for hatching in a shallow rectangular plastic dish (13cm x 8cm x 5cm) filled with 320 ml of a 3.3% solution of artificial sea water. The box had been divided into two unequal compartments with several 2 mm holes plastic divider clamped to the box. About 5.0 g (approximately 300 brine shrimp eggs) were sprinkled onto the bigger compartment of the box. About 0.5 g of yeast was added and covered to this bigger compartment only. On the smaller compartment of the box, lit lamp was hanged to attract the hatched larvae through the perforations into the smaller compartment. Hatching took place after about 48 hours.

3.5.4 Bioassay of *Artemia salina*

For acute toxicity tests, the assay was performed as previously described by Meyer *et.al.* (1982). 10 *Artemia salina* nauplii were transferred into each sample vial (five replicates

for three serial dilutions 5, 50, 500 μl and the control) using 230 mm disposable glass Pasteur pipettes and filtered brine solution was added to make 5 ml. The nauplii were counted macroscopically in the stem of the pipette against a lighted background (Figure 5).

Glass Pasteur pipette stem

Petri dish containing *A. salina* nauplii
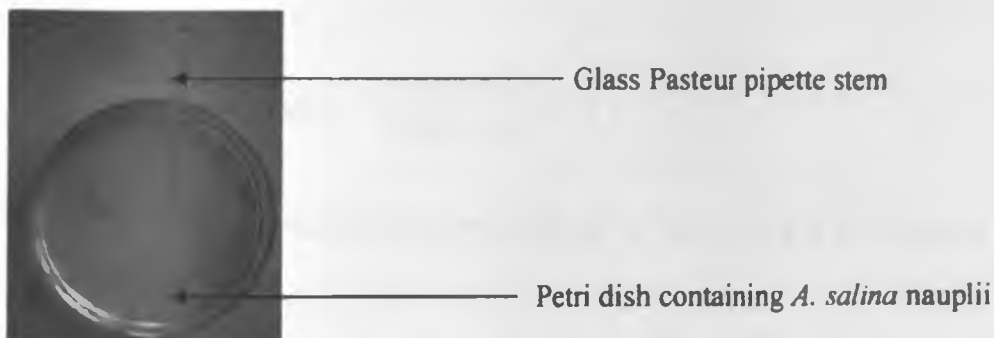
Figure 5: A photograph showing count of *Artemia salina* nauplii along a stem of glass Pasteur pipette.

A drop of dry yeast suspension (Red star; 3 mg in 5 ml artificial sea water) was added as food to each vial. All the vials were maintained under illumination (Figure 6).
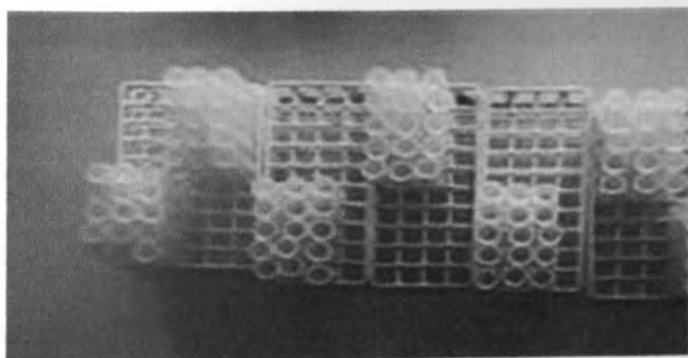
Figure 6: A photograph showing six sets of vials in which each vial has ten *Artemia salina* nauplii inoculated. 5 replicates for three serial dilutions for five different aqueous plant extracts; 1 set treated with distilled water.

Surviving nauplii were counted with the aid of a 3x magnifying glass, after 24 hours, and the percentage of deaths at the three dose levels and control determined. In cases where control deaths occurred, the data was corrected using the formula by Abbott (1925) for control mortality as follows:

$$\% \text{ death} = \frac{\text{test-control}}{100\text{-control}} \times 100$$

The surviving nauplii were killed by the addition of 100 µl of 5% (v/v) phenol to each vial.

3.5.5 LC_{50} determinations

The lethal concentration fifty (LC_{50}), 95% confidence interval and slope were determined from the 24 hour counts using the Finney (1971) computer programme.

3.6 Data and statistical analysis

Data was analyzed using one-way ANOVA (GenStat, 13th ed.) to test for significant difference between mean results obtained for different samples, and Dunnetts test was used for multiple comparisons of significance between results of the same sample means against controls (Graph Pad Instat V.2.04). Values with $p \leq 0.05$ were considered significantly different.

CHAPTER FOUR: RESULTS

4.1 Efficacy of plant extracts against *in vivo Plasmodium berghei* infection

Five crude extracts from different plant species were evaluated in the current study. *Plasmodium berghei* - infected mice were monitored for eleven days and number of surviving mice recorded (Table 2). One mouse treated with *D. cinerea* aqueous root extract died before 4-day suppressive test was complete. By day seven post infection, all mice treated with distilled water had died. Only one mouse treated with *Acacia seyal* aqueous root extract survived up to day eleven (11) post infection. Counting was done.in the morning of each day.

Table 2: The number of surviving mice over 11 days period after treatment with different plant extracts

Treatment	Surviving Mice											
	0	1	2	3	4	5	6	7	8	9	10	11
Distilled water	5	5	5	5	4	1	1	0	0	0	0	0
A. indica	5	5	5	5	4	2	1	1	0	0	0	0
A. seyal	5	5	5	5	3	3	3	2	1	1	1	1
D. cinerea	5	5	4	4	4	3	2	2	1	0	0	0
G. trichocarpa	5	5	5	5	5	4	3	2	1	1	1	0
T. indica	5	5	5	5	5	4	3	1	0	0	0	0
Chloroquine	5	5	5	5	5	5	5	5	5	5	5	5

There were varied effects of the aqueous extracts of different plant species against chloroquine sensitive (D6) *Plasmodium berghei* clones. Parasite density ranged from

45.0% and 67.9% among plant extract-treated infected mice (Table 3). The level of parasitaemia was highest in the negative control at 70% and lowest in the positive control in which all parasites were cleared.

Table 3: Percentage parasitaemia in *Plasmodium berghei* infected mice treated with five aqueous plant extracts, distilled water and chloroquine.

Treatment	Aqueous plant extracts					Distilled	Chloro-
	Azadirachta indica	*Dichrostachys cinerea*	*Tamarindus indica*	*Acacia seyal*	*Grewia trichocarpa*	Water	quine
Mouse 1	68.2	-	49.8	48.9	41.8	74.3	0.0
Mouse 2	67.5	65.4	51.6	50.0	46.4	69.7	0.0
Mouse 3	67.9	64.2	53.4	52.9	42.9	70.4	0.0
Mouse 4	68.0	65.9	45.2	46.6	45.9	64.4	0.0
Mouse 5	67.7	66.8	62.0	54.4	47.8	71.2	0.0
Mean	67.9	65.5	52.4	50.5	45.0	70.0	0.0

Chemosuppression ranged between 3.1% and 35.8%. Chemosuppression in the negative control was not determined. One mouse treated with *Dichrostachys cinerea* aqueous root extract died before 4-day suppressive test was complete (Table 4). Chloroquine was the most effective whereby it cleared all parasites.

Table 4: Percentage Chemosuppression in *Plasmodium berghei* infected mice treated with five aqueous plant extracts, distilled water and chloroquine.

Treatment	Aqueous plant extracts					Distilled Water	Chloro-quine
	Azadirachta indica	*Dichrostachys cinerea*	*Tamarindus indica*	*Acacia seyal*	*Grewia trichocarpa*		
Mouse 1	2.6	-	28.8	30.2	40.3	N/A	100.0
Mouse 2	3.6	6.6	26.3	28.6	33.8	N/A	100.0
Mouse 3	3.0	8.2	23.8	24.4	38.7	N/A	100.0
Mouse 4	2.9	5.8	35.5	33.5	34.4	N/A	100.0
Mouse 5	3.3	4.5	11.5	22.3	31.7	N/A	100.0
Mean	3.1	6.3	25.1	27.8	35.8	N/A	100.0

There was significant ($p \leq 0.05$) difference on percentage parasitaemia between treatment with aqueous plant extracts and chloroquine. The highest parasitaemia was recorded when model mice were treated with *A. indica* root extract (Figure 7). There was no significant difference ($p \leq 0.05$) in parasite density among sterile distilled water, *A. indica* and *D. cinerea* treated mice; and parasitaemia between *A. seyal* and *T. indica* were not significantly different. The lowest parasite density was observed when test mice were treated with *G. trichocarpa* aqueous stem bark extract among the plant extracts. Chloroquine cleared all parasites.
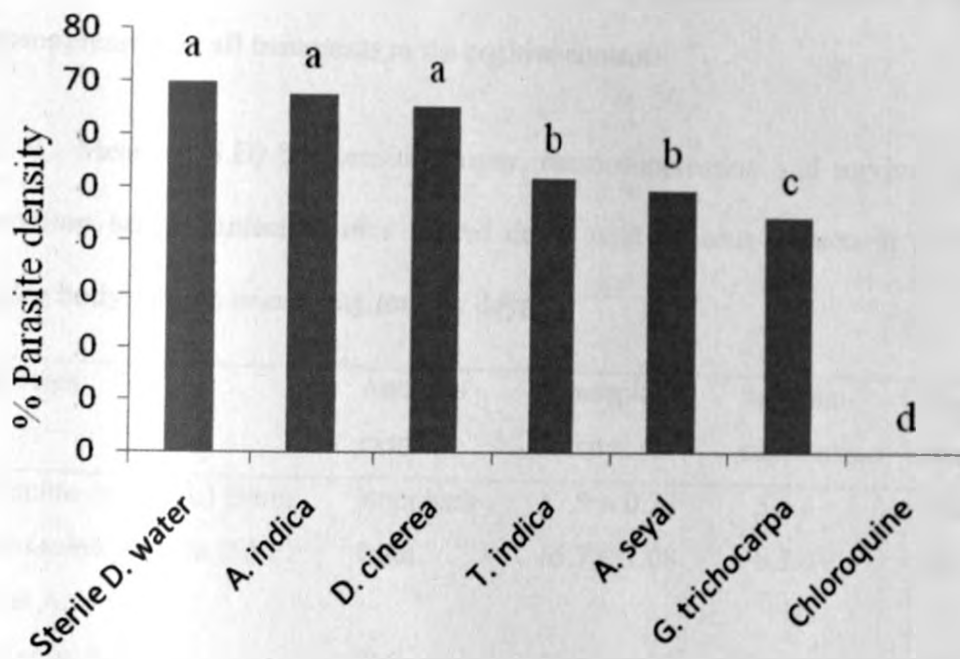
Figure 7: Effects of plant extracts on parasite density of *P. berghei* –infected Swizz mice.

Mice treated with *Azadirachta indica* root extract showed the highest parasite density of 67.9% and lowest chemosuppression of 3.1% (Table 5). Mice treated with *Grewia trichocarpa* root extracts showed the lowest mean parasite density of 45.0% and the highest chemosuppression of 35.8%. There was significant difference ($p \leq 0.05$) between chemosuppression produced by chloroquine to that of five aqueous plant extracts. *Grewia trichocarpa* suppressed parasitaemia that was significantly different ($p \leq 0.05$) to that of the other four plant extracts. On day seven post infection, when there was 100% mortality of mice treated with sterile distilled water, extracts from three plant species *Acacia seyal, Dichrostachys cinerea, Grewia trichocarpa* maintained a survival rate of 40% while extracts from two plant species *Azadirachta indica* and *Tamarindus indica* had a survival rate of 20%. Root extracts produced a longer (40%) survival rate but root and stem bark

extracts gave a shorter (20%) survival rate. There was significant difference ($p \leq 0.05$) chemosuppression in all treatments to the positive control.

Table 5: Mean (± S.D) % parasite density, chemosuppression and survival time of *Plasmodium berghei* infected mice treated orally with aqueous extracts at a dose of 10 mg/kg body weight, once a day for four days.

Plant species	Aqueous extract	%parasite density	%chemo-suppression	%survival (on day 7)
Azadirachta indica (L) Burm	Root bark	67.9 ± 0.27	3.1 d	20.0
Dichrostachys cinerea (L) Wight et Arn	Root	65.7 ± 1.08	6.3 d	40.0
Tamarindus indica L.	Stem bark	52.4 ± 6.17	25.1 c	20.0
Acacia seyal Del.	Root	50.5 ± 3.12	27.8 c	40.0
Grewia trichocarpa Hochst ex A.Rich.	Root	45.0 ± 2.51	35.8 b	40.0
Chloroquine [a]	-	0.0 ± 0.00	100.0 a	100.0
Distilled water[b]	-	70.0 ± 3.59	N/D	0.0

[a] – Positive control; [b] – Negative control; N/D – Not determined.

4.2 Bioactivity of plant extracts on *Artemia salina* larvae

Plotting of mortality percentage *versus* log of concentration for all plant extracts demonstrated that there was a direct proportional relationship between the concentration of the extracts and the degree of lethality (Figure 8).
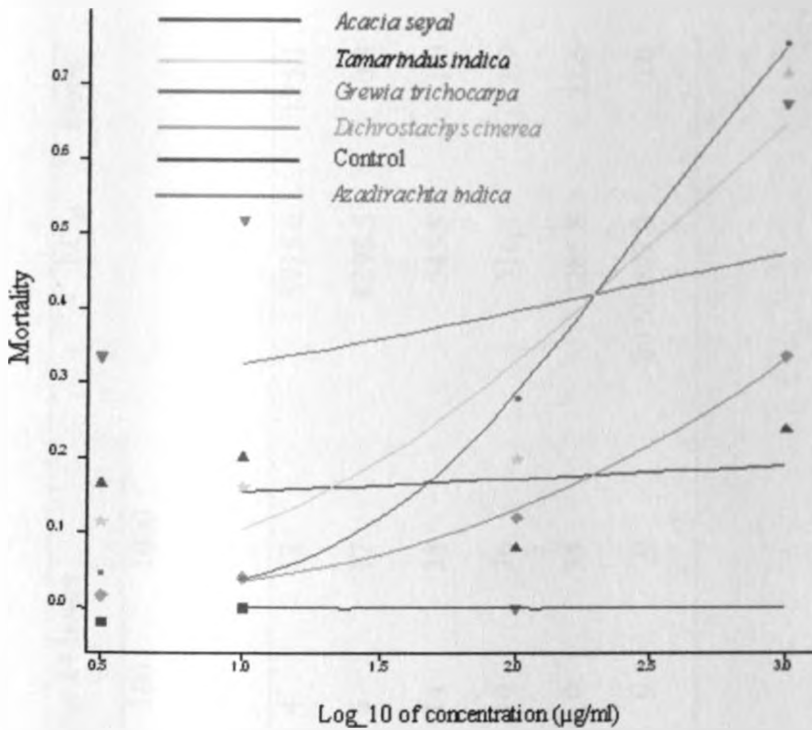
Figure 8: Acute toxicity of different aqueous plant extracts against brine shrimp larvae after 24 hr exposure.

In the evaluation for general toxicity using brine shrimp, maximum mortalities took place at a concentration of 1000 µg/ml whereas least mortalities were at 10 µg/ml concentration. No mortality was observed during the 24 hours in the control (0.0 µg plant extract/ml) and there was no 100% mortality rate achieved at the three concentrations tested. One plant extract, *Azadirachta indica* had an LC_{50} (mouse) of 285.8 µg /ml, and was considered moderately toxic. Two plant extracts, *Tamarindus indica* and *Grewia trichocarpa* and had an LC_{50} of 516.4 and 545.8 µg/ml respectively and were considered weakly toxic while the crude plant extracts of *Acacia seyal* and *Dichrostachys cinerea* had acute toxicity of 1000 and above and were therefore non-toxic (Table 6)

Table 6: Acute toxicity of five aqueous crude plant extracts at different concentrations against *Artemia salina* larvae.

Family	Plant species	% Death after 24 hours			LC_{50}	Slope
		10 µg/ml	100 µg/ml	1000 µg/ml		
Mimosaceae	*Acacia seyal* Del.	10	4	12	5915.6	175.1
Mimosaceae	*Dichrostachys cinerea* (L.) Wight & Arn.	2	6	17	8298.5	0.8
Tiliaceae	*Grewia trichocarpa* Hochst ex A.Rich.	2	14	38	545.8	1.0
Caesalpiniaceae	*Tamarindus indica* L	8	10	36	516.4	1.7
Meliaceae	*Azadirachta indica* (L) Burm	26	0	34	285.8	22.6
N/A	Distilled water[a]	0	0	0	80723503.0	0.0

N/A- Not determined; [a] - Control

CHAPTER FIVE: DISCUSSION

The current study was carried out to investigate the *in vivo* antimalarial activity and acute toxicity of five medicinal plants commonly used to treat malaria by the Digo community of Msambweni district. Data was collected to validate the ethnopharmacological utilization of the various plants and the anecdotal efficacy associated with the collected species by the study community.

Extracts with a high chemosuppression for the parasites offer a potential source for new antimalarial drugs. The mean percentage parasitaemia in the negative control was 70.0% which was the highest witnessed in all the groups treated with the various plant extracts. This indicated that most of the analysed crude extracts had *in vivo* antimalarial activity and did suppress the multiplication of *P. berghei* parasites in mice. Chloroquine, the drug of choice for the treatment of malaria, cleared all the parasites, while parasitaemia in the negative control increased with time, culminating in the death of all mice by day seven post infection.

Mice treated with *Grewia trichocarpa* root extract showed the highest chemosuppression of 35.8%. These findings were in agreement with literature data of other *Grewia* species. Ma *et al.* (2006) demonstrated that some triterpenoids e.g. 3α, 20-lupandiol, grewin, nitidanin and 2α, 3β-dihydroxy-olean-12-en-28-oic acid isolated from *G. bilamellata* are responsible for the antimalarial effect and showed varying degrees of *in vitro* activity against *P. falciparum*. A new coumarinolignan was isolated from a sample of *Grewia*

bilamellata Gagnep. (Tiliaceae) (Ma *et al.*, 2006). Chemosuppression is inversely related to parasitaemia and plant extract which was shown to reduce parasitaemia to low level showed high chemosuppression ability. This may partially validate ethnomedical use of this herb in management of malaria. *In vivo* antiplasmodial activity of plant extract is a better screening method compared to *in vitro* method because some drugs act as febrifuges, prodrugs, or immuno-modulators (Muregi *et al.*, 2004). It can be predicted that it can produce a better recovery of symptomatic malarial combination with other antimalarial that have synergy with it especially due to fact that it is prescribed with other plant extracts among Msambweni community traditional healers (Nguta *et al.*, 2010).

Root extract of *A. seyal* had chemosuppression of 27.8%. This results is in agreement with the bark ethanolic extract of *Acacia fernesiana* that exhibited activity against *Plasmodium berghei* with inhibition of 32% (Garavito *et al.*, 2006). According to Doughari (2006), the plant water extract of the fruits has shown tannins, saponins, sesquiterpenes, alkaloids and phlobatannins that have been implicated to have antiplasmodial activity. However, the use of root extract has been documented for the first time. This results need re-affirmation to validate or disqualify monotherapy of symptomatic malaria with this aqueous plant extract. Other studies, however, have shown that other *Acacia* species, for example *Acacia tortilis* root bark possess antimalarial activity of IC_{50} of >10.0 µg/ml (Koch *et al.*, 2005) and would not be considered for follow up as an antimalarial candidate.

Chemosuppression produced by a plant extract is associated with its inherent ability to clear parasite in infected cells. Inability of a plant extract to completely clear parasites in infected cells may be due to increased biotransformation of the crude plant extract. Stem-bark extraction of *Tamarindus indica* showed chemosuppression of 25.1% which was significantly different from that of negative control. Similar results for the methanol extract of the *T. indica* stem bark showed moderate activity with an IC50 value of 10 mg/ml on 3D7 *Plasmodium berghei* strain (Ahmed *et al.*, 1999). *T. indica* extracts stimulate the lymphocyte proliferation in the presence or absence of phytohaemagglutinin (PHA) stimulator. This plant is widely used in folk medicine to treat many infectious diseases including malaria. This might be by stimulation of the immune system leading to a reduction in the level of parasitaemia so that natural immunity can cope with these infections (Ahmed *et al.*, 1999).

Root extract of *Azadirachta indica* showed 3.1% chemosuppression. It was the lowest value recorded among tested plant extracts. *Azadirachta indica* is the third commonly used herbal medicine to treat malaria in Kenya after *Ajuga remota* and *Caesalpinia volkensii* (Kuria *et al.*, 2001). Efficacy of the extract is attributed to Azadirachtin, a limonoid of highly oxygenated terpenoids (Roi *et al.*, 2006). Many other communities around the world use these plants as traditional anti-malarials. However, activity depends on many factors such as the season in which the plant is collected, the age of the plants, intraspecies variation, the environmental conditions part collected among others (Muregi *et al.*, 2004). Azadirachtin (a tetranortriterpenoid) has been detected in all aerial parts of neem plant with concentration decreasing in the order of mature seed kernel,

leaves, bark, roots and stem (Sundaram, 1996). Lack of significant antimalarial effect of the root extract on the other hand is an advantage for it would allow sustainable harvesting of the plant.

In vivo studies in mice with *Plasmodium berghei* have been uniformly disappointing (Obih and Makinde, 1985). Many reasons could explain these poor *in vivo* activities. It may be that mice do not metabolise Neem extracts in the same way as humans. Murine *Plasmodium* have different properties and sensitivities compared with human *Plasmodium falciparum* and the 4-day suppressive test may be not sufficient to evaluate plant extracts (Willcox and Bodeker, 2004).

The aqueous root extract of *D. cinerea* had no significant effect in treatment of symptomatic malaria and would not make as a good follow for discovery of malarial herbal drug.

If administration of drug is not continued before the host has recovered from infection, the parasite may regain virulence to a level to overcome host's immune system leading to death. Extracts of *Acacia seyal, Dichrostachys cinerea* and *Grewia trichocarpa* produced highest survival of 40%. Lower survival of mice treated with *A. indica* could partly be contributed by traces of toxins. *T. indica* gave a lower survival of 20% probably due fact that it was weakly toxic. Interestingly though *A. seyal* gave a high survival of 40%, it was non-toxic. More so, it had one mouse surviving on day eleven of post infection when all

plant extract-treated mice had died. Further investigation may be recommended to find out bioactive compound that maintains high survival.

LC_{50} is the concentration of the test compound that causes 50% mortality among the test organism during a certain exposure time. The *in vivo* lethality in a simple zoological organism, such as the brine shrimp lethality test (BST), developed by Meyer *et al.* (1982) can be used as a simple tool to guide screening and fractionation of physiologically active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive. This assay is considered as a simple, low-cost, highly sensitive, efficient rapid, bench top acute toxicity screening assay which requires only a relatively small amount of sample (2-20 mg). Since it has a good correlation acute toxicity, it has been extensively used as a preliminary tool for screening of acute toxicity of plant crude extracts and different fractions of crude extracts (Kaur *et al.*, 2009).

This general bioassay detects a broad range of biological activities and a diversity of chemical structures. One basic premise here is that toxicology is simply pharmacology at a higher dose, thus if we find toxic compounds, a lower, non-toxic, dose might elicit a useful, pharmacological, perturbation on a physiologic system (McLaughlin, 1991). In general, the smaller the LD_{50} value, the more toxic the chemical is. The opposite is also true: the larger the LD_{50} value, the lower the toxicity.

The best extract against CQ-sensitive *P. berghei* was that of *Dichrostachys cinerea* root extract which gave LC_{50} of 8298.5 µg/ml. *Acacia seyal* scored second among non-toxic plant extract (LC_{50} 5915.6 µg/ml). Therefore, *D. cinerea* and *A. seyal* pose no threat as anti-malarial drug by community that use them for chemosuppression observed can be attributed to having potential to inhibit growth of *Plasmodium* parasites which is not related with toxicity. Screening aqueous extracts of *T. indica* and *G. trichocarpa* showed LC_{50} of 516.4 and 545.8 µg/ml which were considered weakly toxic. It can be argued out that chemosuppression observed was partly due to traces of toxins in the extract but largely due to the extract's ability to elicit antiplasmodial activity. The extract of *A. indica* produced LC_{50} of 285.8 µg/ml and was considered moderately toxic. Partly, the phytoconstituents in the crude extract could be attributed to death of *Plasmodium*-infected red blood cells. More so, presence and concentration of bioactive ingredients usually vary with the age of the plant, intra-species variation and time of the season, locality, extraction method used and length of storage time (Muregi *et al.*, 2004).

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Among the investigated plants, two were non-toxic and two weakly toxic and one moderately toxic to *A. salina* larvae. Interestingly, *A. seyal* was non- toxic, had chemosuppression significantly different to that elicited by sterile distilled water and showed a longer survival rate. Further research may be recommended to establish the cause of this observation. *G. trichocarpa* though was weakly toxic, had a longer survival rate (40%) and a higher chemosuppression. *D. cinerea* aqueous root extract was a poor antimalarial herb of choice despite that it was non-toxic. The results observed suggest that the monotherapy of malaria with aqueous extracts of plant parts under investigation was non- effective. Anecdotal efficacy reported by the study community could be related to synergism of phytoconstituents since the assayed plant parts are used in combination with others to treat malaria. It is also evident that none of the screened plant extracts is toxic to the arthropod invertebrate, *Artemia salina* L. (Artemiidae) larvae, justifying the continued use of the plant parts to treat malaria. Future work is suggested to evaluate the antimalarial activity of organic extracts of the studied plant parts.

6.2 Recommendations

1. The use of the studied plant part extracts should only be used in combination with other herbs for effective treatment.

2. Further research should be carried out to determine the efficacy of aqueous extracts of different plant parts of the plant species to determine their antimalarial effects and acute toxic effect.

3. Plant extracts of *G. trichocarpa, A. seyal* and *T. indica* investigated in this study should further be screened in combination with other plants that have been shown to possess high antimalarial potency to determine the mode of synergy they would exhibit.

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APPENDICES

Appendix 1: Analysis of variance of parasite density in Swizz mice treated with five

different aqueous plant extracts, distilled water and Chloroquine against

Plasmodium berghei

Variate: Parasitaemia

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	17220.32	2870.05	284.91	<.001
Residual	27	271.98	10.07		
Total	33	17492.30			

Stratum standard errors and coefficients of variation

Variate: Parasitaemia

d.f.	s.e.	cv%
27	3.174	6.4

Appendix 2: Analysis of variance of Chemosuppression produced by different plant

extracts and Chloroquine against *Plasmodium berghei* in Swizz mice

Variate: %_Chemosuppression

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	5	30231.84	6046.37	309.17	<.001
Residual	23	449.81	19.56		
Total	28	30681.65			

Stratum standard errors and coefficients of variation

Variate: %_Chemosuppression

d.f.	s.e.	cv%
23	4.422	13.0

Tables of means % chemosuppression of *Plasmodium berghei* (D6) in Swizz mice

Grand mean 33.941

Treatment	*A. indica*	*A. seyal*	*D. cinerea*	*G. trichocarpa*	Chloroquine	*T. indica*
Mean	3.1	27.8	6.3	35.8	100.0	25.2
Replicates	5	5	4	5	5	5
s.e	1.978	1.978	2.211	1.978	1.978	1.978

Appendix 3: Regression analysis for acute toxicity of aqueous plant extracts against

brine shrimp larvae

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx chi pr
Regression	10	192.67	· 19.27	19.27	<.001
Residual	5	79.42	15.88		
Total	15	272.09	18.14		

Appendix 4: Estimated effective doses (µg/ml) of different aqueous plant extracts at

which it caused 50% death of brine shrimp larvae

Group	LC	estimate (Log_10)	s.e.	lower 95%	upper 95%
Acacia seyal	50.00	3.772	0.298	3.279	4.430
Azadirachta indica	50.00	2.456	0.207	2.081	2.874
Control	50.00	7.907	5.717	-2.630	19.203
Dichrostachys cinerea	50.00	3.919	0.313	3.404	4.613
Grewia trichocarpa	50.00	2.737	0.219	2.352	3.191
Tamarindus indica	50.00	2.713	0.218	2.329	3.164