ANTI-MALARIAL ACTIVITIES AND TOXICITY LEVELS OF EXTRACTS OF FOUR SELECTED MEDICINAL PLANTS USED IN PARTS OF KENYA

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DECLARATION

Declaration by Candidate

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DEDICATION

I dedicate this work to my parents for their support since the beginning of my studies, and my brothers and sisters for their love and moral support.

ABSTRACT

Malaria is a life threatening vector borne disease and with a long history in Africa South of Sahara. Conventional drugs have been used to manage malaria only during the last 5 decades. The spectacular results of western medicine and medical teaching based upon it led to devaluation of the use of herbal medicine in malaria treatment and associated science-driven research and developement on this medicine. On the other hand, resistance development to most of the existing malaria drugs, and recently to the first line Artemisinine based drug in Cambodia, and the increasing burden of the disease in Africa, demand a fresh look at the potential of traditional medicines. Significantly, crude preparations from different communities in malaria endemic areas have been in use in Kenya. The main aim of this study was to determine the efficacy and safety of plant extracts from four selected medicinal plants used in Kenya for treatment of malaria. To achieve this, crude extracts from four medicinal plants with traditional reputation were extracted using methanol and water solvents. Their yields were determined and then, they were screened for their *in vitro* antiplasmodial activity using a radioisotopic uptake of hypoxanthine against chloroquine sensitive D6 strain and the Chloroquine resistant W2 strain of *Plasmodium falciparum*. They were also screened for their antimalarial activity *in vivo* in a 4 day suppressive test against *Plasmodium berghei* ANKA. Animal acute toxicity and cell cytotoxicity using VERO E 99 cells was also carried out for all the extracts. It was found out that all the crude water extracts were significantly (P=0.023) more than the crude methanol extracts and were all active against chloroquine sensitive D6 strain of *Plasmodium falciparum*. Aerial parts of *Fuerstia africana*, methanol extract had the highest activity (IC_{50} , 1.841± 0.82 µg/ml) followed by stem bark of *Ximenia americana*, methanol extract (IC_{50}, 2.108 µg/ml) and the roots of *Sericocomopsis hilderbrandtii* (IC_{50}, 2.12 µg/ml) against D6 strain of *Plasmodium falciparum.* All water extracts also had high antiplasmodial activities against D6 strain. Methanol extracts of *Sericocomopsis hilderbrandtii* aerial parts, roots, *Pentas lanceolata* aerial part and *Ximenia americana* stem bark had moderate antiplasmodial activity against W2 strain of *Plasmodium falciparum*. Water extracts of *Pentas lanceolata* aerial part and *Fuerstia africana* also had moderate activities against chloroquine resistant W2 strain of *Plasmodium falciparum*. The roots and aerial parts of *Sericocomopsis hilderbrandtii* and stem bark of *Ximenia americana* had low antiplasmodial activities against W2 strain of *Plasmodium falciparum.* Of the ten extracts that were tested *in vivo* seven showed good anti-malarial activity with Chemosuppresion ranging from 30.29% to 64.92% while three demonstrated low activity. There was significant difference (P=0.012) in the parasite density between the mice treated with the active plant extracts and the untreated controls. Whereas most of the plant extracts were not cytotoxic at 100µg/ml, *Fuerstia africana* was moderately toxic with CC_{50} at 63.45µg/ml. All the extracts tested for acute toxicity were safe with LD_{50} > 5000mg/Kg. The confirmed activity activity of most of these plants extracts and their lack of toxicity in mice and in VERO E 99 cells may partially validate their use as antimalarials by some Kenyan communities. It was therefore recommended that some of these extracts should be further evaluated for large scale production, formulation and use.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinine Combined Therapy
CBRD	Center for Biotechnology Research and Development
CDC	Centers for Disease Control
CTMDR	Center for Traditional Medicine and Drug Research
CQ	Chloroquine
DMSO	Dimethy Sulphoxide
FBS	Fetal Bovine Serum
g/l	Grams per litre
IPR	Intellectual Property Rights
KEMRI	Kenya Medical Research Institute
MEM	Minimum Essential Medium
MTT	Thiazolyl Blue Tetrazolium Bromide
$NaHCO_3$	Sodium Bicarbonate
THPs	Traditional Herbal Practitioners
TNF	Tumour necrosis factor
µl	Microlitre
USD	United States Dollars
WHO	World Health Organization

OPERATIONAL DEFINITION OF TERMS

ad libitum: Constant provision of food and water to mice making them available at their disposal.

Antimalarial activity: The ability of plant extracts to inhibit growth of malaria parasite in mice.

Antiplasmodial activity: The ability of plant extracts to inhibit growh of *plasmodium* parasite in cells.

Artemisinine Combined Therapy (ACT): Combination of Artemisinine derivatives with other antimalarial drugs

Chemosuppression: The suppression of parasite growth by plant extracts

Chemotherapy: The use of drugs to eradicate a disease

Cryopreservation: The process whereby parasites are preserved in liquid Nitrogen for future use

Parasite density: The parasite load in blood of of a swiss albino mouse determined through microsopy

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is a life threatening vector borne infection caused by a protozoan parasite belonging to the genus *Plasmodium.* There are four *Plasmodium* species that infect humans. They are *P. falciparium, P. ovale, P. malariae* and *P. vivax* and they can be distinguished by their morphology (Mendis, *et al.,* 2001). *P. vivax* and *P. falciparium* are the most prevalent species worldwide, *P. malariae* are rare and not lethal but commonly found in parts of Africa. *P. falciparium* causes severe malaria leading to high incidence of mortality (Daily, 2006). An estimated 3.4 billion people are at risk of malaria 1.2 billion of whom are at high risk (WHO, 2014). In 2012 alone there were an estimated 207 million cases of malaria globaly, and an estimated 627 000 deaths with 90% of all these malaria deaths occurring in sub-Saharan Africa (WHO, 2013). According to WHO (2013) an estimated 482 000 children under five years of age were killed by malaria, which is equivalent to 1300 children per day or one child every minute.

Malaria negatively affects cognitive and physical development, creates complications in pregnancy, causes chronic debilitation and can result in anaemia (Holding and Snow 2001). These adversely affects the the social and economic development of the affected countries (Sachs and Malaney, 2002, Breman *et al.,* 2004, Malaney *et al.,* 2004), which are mainly in Tropical Africa (Trape, 2002).

Key strategies in the control of malaria include Chemotherapy, indoor residual spraying, and the use of insecticide-treated nets (Anon, 1993). Unfortunately, these control strategies are becoming less effective with the rapid development and spread of resistance to widely used drugs. Plants have always been considered to be a possible alternative and rich source of new drugs (Willcox, 1999). Excellent antimalarial drugs such as quinine and artemisinine were obtained from plants (Basco *et al.,* 1994; Kayser *et al.,* 2003; Muthaura, 2007). So far reports of resistance to Artemisinine derivatives like artesunate have emerged in western Cambodia (Noedl *et al.,* 2010, Dondorp *et al*, 2009). This is a threate to global control of malaria (Dondorp *et al.,* 2010) since other antimalarial drugs such as, chloroquine and sulfadoxine pyrimethamine have already succumbed to resistance (White, 2010).

Drugs derived from natural products have fewer side effects than synthesized molecules (Breman *et al* 2004). In Africa traditional medicinal plants are frequently used to treat malaria (Gessler *et al*., 1995; Kokwaro 1993; Koch, 2005). In the effort for search for a new potent antimalarial drug from traditional medicinal plants, four medicinal plants, used to treat malaria in Kenya were targeted. These included *Sericocomopsis hildebrandtii* Schinz, *Pentas lanceolata* (Forssk.) Defleurs, *Fuerstia africana* T.C.E. Friers, and *Ximenia americana* L. The antimalarial and toxicity activities of these four medicinal plants were investigated.

1.2 Statement of the problem

Malaria remains a threate to the life of people especially children and pregnant mothers (Trape, 2001). This posses a big public health and economic challenge (Trape, 2002).

Efforts to contain malaria are set back by the emergence and spread of drug resistant *Plasmodium falciparun* (Breman, 2004; Trape, 2002). *Plasmodium falciparum*, the cause of human malaria is becoming increasingly resistant to standard antimalarial drugs (Muregi *et al.,* 2007). Resistance to once very effective drugs such as chloroquine, mefloquine, pyrimethamine sulfadoxine (SP) and quinine have been reported (Pickard and Wernsdorfer, 2002). Reports of reduced *in-vivo* susceptibility of *P. falciparum* to artemisinine derivatives like artesunate have emerged in western Cambodia (Noedl *et al.,* 2008; Dondorp *et al.,* 2009, 2010; Noedel *et al.,* 2010). This trend added to the fact that there is still no obvious class of antimalarial agents in the pipeline ready to replace Artemisinine (Wongsrichanalai and Meshnick, 2008) is worrying. Therefore there is an urgent need for development of new antimalarial drug that will overcome the drug resistance.

1.3 Justification

Medicinal plants have been used since time immemorial as a source of medicines for mankind. Antimalarial drugs like Artemisinine and Quinine originated from plants (Basco *et al*., 1994; Kayser *et al*., 2003). Herbal medicines have few or no side effects at all and are less susceptible to parasite resistance. It is therefore important to look at the viability of herbal medicine with the aim of coming up with drugs that will overcome resistance. Convetional antimalarial drugs are expensive and may not be readily accesible in the rural setting. In Kenya Medicinal plants have been used to treat malaria. However these medicinal plants may have other effects in the body other than antimalarial activity that suppress the symptoms of malaria. These may be antiinflamatory, analgesic or

antipyretic effects which are all symptoms of malaria. Despite their long period of use in Kenyan as antimalarials, the safety and efficacy of these medicinal plants has not been established. This study seeks to validate the efficacy and safety of four medicinal plants used in Kenya.

1.4 Objectives

1.4.1 Main Objective

To determine yields and antimalarial activities and toxicity levels of plant extracts from four selected medicinal plants used in Kenya.

1.4.2 Specific Objectives

1. To screen crude extracts of *P. lanceolata, F. africana, X. americana* and *S. hilderbrandtii* for *in vitro* antiplasmodial activity
2. To evaluate the crude extracts of *P. lanceolata, F. africana, X. americana* and *S. hilderbrandtii* for *in vivo* antiplasmodial activity
3. To asses *in vitro* cytotoxicity activity of the extracts
4. To asses *in vivo* acute toxicity of the extracts

1.5 Hypotheses

H_{01}: The methanol and water extracts of *P. lanceolata, F. africana, X. americana* and *S. hilderbrandtii* plants do not have *in vitro* antiplasmodial activity.

H_{02}: The crude extracts of *P. lanceolata, F. africana, X. americana* and *S. hilderbrandtii* do not have *in vivo* antimalarial activity

H_{03}: The crude extracts of *P. lanceolata, F. africana, X. americana* and *S. hilderbrandtii* do not have *in vitro* cytotoxicity activities

H_{04}: The crude extracts of *P. lanceolata, F. africana, X. americana* and *S. hilderbrandtii* do not have *in vivo* acute toxicity

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria chemotherapy

Chemotherapy involves the use of chemical agents to fight parasites once they are in the body. Most antimalarial drugs target the asexual erythrocytic stages of the parasite (Ibezim and Uche 2008). Often the parasite degrades hemoglobin in its acidic vacuole, producing free heme able to react with molecular oxygen and generate reactive oxygen species as toxic byproducts (Francis *et al.,* 1997). The newest class of antimalarials is based on artemisinine, its hemi synthetic derivatives and synthetic analogues, whose mode of action is thought to be similar to that of Chloroquine (Luo and Shen 1987).

Development of resistance by the parasite to most conventional anti-malarial drugs is a major set back in malaria control (Olliaro and Bloland, 2001). Development of Drug resistance is contributed by factors such as the use of sub therapeutic doses of drugs, not completing the treatment regimen, self treatment, poor compliance, mass administration, use of drugs with long half-life, and high transmission intensity **(**Ibezim and Uche, 2008). The first line antimalarial drugs recommended for use today include artemisinine and its derivatives. This drug has found very effective use in a short period of time and one advantage they have is that they have short half-life, which counters one of the major factors that lead to drug resistance (Robert *et al.,* 2001). Besides, there has not been any reported case of severe side effects in their use. Other recent antimalarial drugs are

halofantrine (Halfan ®), atovaquine, amodiaquine and mefloquine (Robert *et al.,* 2001; Srivastava *et al.,* 1997; White, 1998).

2.1.1 Chloroquine

Chloroquine (CQ), is a 4-aminoquinoline and has been the mainstay of malarial chemotherapy for much of the past five decades (Foster, 1994). It was first synthesized in 1934 and became the most widely used antimalarial drug by the 1940s (Loeb *et al*., 1946). The success of this drug has been based on excellent clinical efficacy, limited patient toxicity, ease of use and cost-effective synthesis. Importantly, CQ treatment has always been affordable as little as USD 0.10 in Africa (Muregi and Ishih, 2010; Wells, 2010). By 1994 CQ was the third most widely consumed drug in the world after aspirin and paracetamol (Foster, 1994). Mechanisms of action for CQ include the inhibition of protein synthesis (Surolia and Padmanaban, 1991), the inhibition of food vacuole phospholipases (Ginsburg and Geary, 1987), and the inhibition of aspartic proteinases (Vander *et al.,* 1986) in the parasite.

The use of Chloroquine was limited by development of parasite resistance (Kouznetsov and Go´ mez-Barrio, 2009). Chloroquine resistance occurred due to selective pressure as a result of widespread use of this drug to eradicate malaria worldwide (Wellems *et al*., 2001). Resistance to Chloroquine in *P. falciparum* first appeared virtually simultaneously in Southeast Asia (Thai-Cambodian border) and South America (Colombia) in late 1950s (Young, 1996, Wernsdorfer WH, 1991). From there, Chloroquine resistance spread to the

other parts of the world where malaria is endemic (Peters, 1987). Chloroquine resistance was reported in the Eastern Africa in 1978 (Peters, 1987).

Chloroquine resistance in *P. vivax* was noted for the first time in Papua New Guinea then it spread to other parts of the world (Rieckman *et al.*, 1989). From India also there are now several reports of Chloroquine resistance in *P. vivax* (Potkar *et al*., 1994, Garg *et al*., 1995, Dua *et al*., 1996). Resistance in *P. vivax* is more serious as hypnozoites will cause relapse of resistant parasites and *P. vivax* is a mixture of various strains with respect to incubation period, relapsing pattern and response to primaquine (Adak *et al*., 1978) since sulpha drugs are not effective in its treatment (Ibezim and Uche 2008).

2.1.2 Suphadoxine-pyrimethamine (SP)

Pyrimethamine and Suphadoxine are antifolate drugs that target *dhfr* and *dhps* genes respectively (Sibley *et al*., 2001). Its resistance has been reported from large parts of South-East Asia, Southern China and Amazon basin (Aramburu *et al*., 1999). In Africa, SP resistance was detected in the late 1980s which has since spread more in the east than in the West. Sulfadoxine pyrimethamine is currently used as the first line drug in Kenya (Dondrop *et al*., 2005)

2.1.3 Quinine

Quinine was derived from the bark of Cinchona spp, and was introduced in Europe from South America in the 17^{th} century (Ward *et al*., 1995). The first case of quinine resistance was reported from South America nearly a century ago (Zalis *et al.*, 1998). Resistance to

it was observed from Thai-Cambodia border in mid 1960s (Pickard and Wermsdofer, 2002). The clinical resistance to quinine therapy was noticed sporadically in Southeast Asia and Western Oceania. It is less frequent in South America and Africa (Jelinek *et al*., 2001). The wide spread use of quinine in Thailand and in the 1980s could be the reason for development of significant resistance (Wernsdorfer and Payne, 1991).

2.1.4 Mefloquine (MQ)

Mefloquine (MQ) is a α-2-piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol and possesses two asymmetric carbon atoms (Caroll and Blackwell 1974). It has long half life of 14-21 days which probably contributed to the rapid development of resistance (Wongsrichanalai *et al.,* 2001). It is active against intra-erythrocytic asexual forms of the four species of *Plasmodium* that infect humans (Foley and Tilley 1998). Resistance was observed in late 1980s near the Thai-Cambodia border due to its widespread use (Wongsrichannalai et *al.*, 2001, Boudreau *et al.,* 1982). In Southeast Asia, mefloquine resistance has been reversed by combination with artemisinine (Nosten *et al*., 2000). Mechanism of action of the drug is by binding to haem and forming a complex which may be toxic to the parasite (Foley and Tilley 1998). Its use is associated with undesirable side effects on the central nervous system (CNS) such as nausea (Ter Kuile *et al*., 1993). There are more serious side effects such as disturbed sleep, heightened anxiety, hallucinations, depression and acute psychosis (Weinke *et al*., 1991).

2.1.5 Amodiaquine

Amodiaquine (AQ) is a 4-aminoquinoline that has been used widely to treat and prevent malaria (Holmgren, 2007). This drug is more palatable (Olliaro and Mussano, 2003) and less toxic in people infected with HIV as reported by (Coopman, 1993). However the use of AQ has been limited by parasite resistance (Ibezim and Uche., 2008). Nevertheless its combination with sulfadoxine-pyrimethamine (SP) is still efficacious in some areas of Africa where moderate resistance exists (Staedke *et al.,* 2004).

2.1.6 Lumefantrine

Lumefantrine is an aryl alcohol whose oral absorption is highly variable and is improved when taken with fatty foods (Bertram, 2006). The drug has a longer elimination half-life and a low recrudescence rate (Ezzet, 1998). It is mainly availlable in combination with Artemether as a fixed-dose oral combination artemether-lumefantrine (CoArtem ®) in a single oral formulation (AL) (Yeung *et al.,* 2004). AL therefore combines the benefits of the fast onset of action of artemether with the long duration of action and high cure rate of lumefantrine (Yeung *et al.,* 2004). This ACT is highly effective even against multi drug resistant malaria parasites with clearance of the parasites from the blood within 2 days (Price *et al.,* 2006).

2.1.7 Artemisinine and its derivatives

Artemisinine (*qinghaosu),* a sesquiterpene lactone endoperoxide is a natural component of the Chinese medicinal plant Qinghao (sweetworm) or *Artemisia anuua* L (Wayman, 1995). It is a 15-C atom structure with a trioxane ring and a lactone ring and has a

molecular weight of 282g (Webster and Lehnert, 1994). Antimalarial properties of Artemisinine were discovered in 1972 (Luo and Shen, 1987). It was found to have a rapid resolution of fever and parasitaemia (DeVries and Dien, 1996). This drug has a broad stage specificity of action and induces fast parasite clearance (White, 2008).

Artemisinine derivatives have also been found to produce rapid clearance of parasites from the blood (DeVries and Dien, 1996) and a significant effect on gametocytogenesis (Peters *et al.,* 1986; Price *et al*., 1998). Artemisinine-Based Combination Therapy (ACT) is recommended first-line treatment of uncomplicated *Plasmodium falciparum* world wide (WHO, 2010). This combination comprises a fast-acting artemisinine derivative with rapid effect on parasite clearance (Lefevre 1999), and a long-acting drug to prevent recrudescence and development of resistance as described by Dondorp *et al.,* (2005). Advantages of Artemisinne combined therapy include fast onset of action(Lefevre, 1999), prevention of recruidescence (Bertram 2006), high cure rates (White, 1999a) and reduced chances of resistance (White 1999b), there are reports of decline in efficacy of Artemisinine-based combination therapy in some areas particularly in western Cambodia (Noedl et *al.,* 2008; Dondorp *et al.,* 2009).

2.1.8 Artemether

Artemether is an oil-soluble methyl ether derivative of artemisinine that is effective against *P. falciparum* which is resistant to all other operationally used antimalarial drugs (Li *et al.,* 1998). It is available as a fixed-dose combination with lumefantrine (Yeung *et al.,* 2004). According to Mcinotsh and Olliaro, (1998), Artemeter is not hypnozointicidal but it is able to reduce gametocyte carriage. Artemether has a rapid onset of action and is

rapidly eliminated from the plasma with half life of two to three hours (Lefevre 1999). The major advantage of Artemeter apart from parasite clearance is that it is easy to use and that it does not induce hypoglycemia as demonstrated by Van Hensbroak *et al*., (1996).

2.1.9 Artesunate

This is a water-soluble hemisuccinate derivative of artemisinine that is widely used (Byakika-Kibwika, *et al*., 2012). It is a potent blood schizonticide active against the ring stage of the parasites (Skinner, *et al*., 1996). It is ideal for the treatment of severe malaria, including cerebral malaria (Nadjm and Behrens, 2012). It is also active against chloroquine and mefloquine resistant strains of *P. falciparum* (Davis *et al* 2001). It is unstable in neutral solution and is therefore only available for injections as artesunic acid (Dondrop, 2005). The essential part of the mechanism of action of artesunate is the presence of a peroxide bond (Foley and Tilley 1998) which breaks inside the parasite forming singlet oxygen as well as free radicals, thereby exerting a direct cytotoxic effect on the cells and the sub-cellular membranes (Navaratnam *et al*., 2000). Compared to quinine, Artesunate is better tolerated, safer, easier to use (Dondrop, 2005; Kreeftmeijer-Vegter *et al*., 2012), and more effective in parasite clearance (Dondrop *et al.*, 2010). It is mainly administered intravenously and has been recognized by the World Health Organization as the drug of choice for severe *falciparum* malaria (WHO, 2006). However decline in efficacy of artesunate monotherapy has been reported in some parts of South East Asia (Noedl *et al.,* 2008; Dondorp *et al.,* 2009; Maude *et al.,* 2009)

2.1.10 Arteether

Arteether, is a semi synthetic derivative of artemisinine and is an active constituent of the plant, *Artemisia annua* (Klayman, 1985). It is a blood schizonticide and active against all stages of *Plasmodium falciparum* (Balint, 2001). The drug is also used in the treatment of cerebral malaria as well as in the chloroquine resistant cases (Patel *et al.,* 2010). Unfortunately, According to Lin and Miller (1995), it is insoluble in water and therefore its formulation causes difficulties. Moreover, Li *et al* (1998) established its therapeutic efficacy is greatly hampered due to its poor bioavailability since approximately 40% of the drug degrades in the stomach.

2.2 Medicinal plants

The main goal of ethno pharmacology is to discover novel plant-derived compounds, based on the indigenous use as medicinal plants, which can be developed into new pharmaceuticals. Historically, plants have proven to be a major source of drugs (Klayman, 1985), with two of the most widely used antimalarials originating from plants; the quinoline-based antimalarials a modeled on quinine, derived from the bark of the Peruvian *Cinchona L*. tree. The endoperoxide-based antimalarials originated from artemisinine, first isolated from the Chinese herbal medicine *Artemisia annua* (Del Rayo Camacho *et al*., 2000). In light of this historic medical success and the fact that most indigenous people living in malaria endemic areas use traditional medicines to fight this disease, there is every possibility that ethno pharmacological approaches could lead to new antimalarial agents (Phillipson *et al*, 1987). Based on their ethno medical and literature information, the following medicinal plants were selected for this study.

2.2.1 *Fuerstia africana* T.C.E. Friers (Lamiaceae)

Fuerstia africana is a low-growing herb endemic to tropical East Africa (Figure 2.1). It is also a remedy for malaria, a purgative, an anthelmintic and relieves ophthalmic or conjunctivitis. Leaf and stem infusion is drunk as an antidote for snake bites (Kokwaro, 1993). A decoction of *F. africana* is also used by traditional health practitioners in Machakos County in the management of diabetes (Lucia *et al*., 2012). Previous studies on *F. africana* by Kigondu (2011) showed that the $CHCl_3$ from leaves exhibited strong antiplasmodial activity against the chloroquine sensitive strain D6 with IC_{50} of 3.8µg/ml. One compound, known as Ferruginol, has been previously isolated from this plant and this substance presented a strong anti-malarial activity, with an IC_{50} of 1.95µg/ml, but also a cytotoxic activity. Therefore, Ferruginol is not a desirable anti-malarial candidate (Koch *et al.*, 2005)

Figure 2. 1. *Fuerstia africana* plant (Source: Plagens, 2013)

2.2.2 *Sericocomopsis hildebrandtii* Schinz (Amaranthaceae)

Sericocomopsis hildebrandtii is a dwarf shrub mainly found in forest bush land (Figure 2.2). According to Kokwaro, (1993) the juice from the roots of the plant locally known as *Oloiturujilpeles* among the Maasai and *Kinkers* for the Tugen is drunk for purgative effect and dysmenorrhea. Earlier investigation on the plant have exhibited ethyl acetate extract of the aerial parts of *S. hildebrandtii* to have moderate antiplasmodial activity against D6 isolate of *Plasmodium falciparum* with IC_{50} values of 16.51± 0.91 µg/ml (Kigondu, 2011). However, Koch *et al.*, (2005) on the other hand reported the roots of *S. hildebrandtii* ($CHCl_3$ extract) to exhibit higher *in vitro* antiplasmodial activity with IC_{50} value of 3.8µg/ml. This extract has also displayed selectivity for the malaria parasite *P. falciparum* as indicated by a low cytotoxicity (ED_{50} >20 µg/ml) against cultured KB cells.

Figure 2. 2 *Sericocomopsis hilderbrandtii* plant (Source: Bygott, 2002)

2.2.3 *Pentas lanceolata* (Forssk.) Defleurs (Rubiaceae)

Pentas lanceolata is an upright tropical evergreen shrub, 3 - 4 feet tall (Figure 2.3). The juice from the roots of *P. lanceolata* is locally known as *Olkilaki-olkerr* among the Maasai of Kenya and is drunk for malaria and depression (Kokwaro, 1993). In Ethiopia a decoction of roots of *Pentas lanceolata* is also used by the Sheko people of South West Ethiopia to treat urinary tract infection (Mirutse *et al.*, 2010). The rosot of the plant has lately been shown to exhibit high *in vitro* antiplasmodial activity. Petroleum ether extract of *P. lanceolata* have been found to have an IC_{50} value of 58.5 µg/ml Kigondu *et al*., (2011). It has also been shown to display selectivity for the malaria parasite *P. falciparum* as indicated by a lack of cytotoxicity (ED_{50} >20 µg/m) against cultured KB cells (Koch *et al*., 2005). Furthermore ethanolic extract of flowers of *P. lanceolata* have been reported to have wound healing activity (Nayak *et al.*, 2005 b).

Figure 2. 3 *Pentas lanceolata* plant (Source: Tortuga, 2008)

2.2.4 *Ximenia americana* L (olacaceae)

Ximenia americana L. (Olacaceae) is a shrubby tree up to 5 m high (Figure 2.4) which is widely distributed in Africa, tropical America and Asia (Burkill, 1997). In Kenya it is widely used to treat different ailments. *X. americana* adopts different names in different communities in Kenya.The Maasai call it Engamai. The Kamba people refer to it as Kitula or Mutula the Luo community on the other hand call it Olemo where as among the Nyankore it is known as Omuseka. Kokwaro (1993), has reported that the fruits of *X. americana* which are edible can be eaten in large quantites to act as a cure for tonsillitis and mouth sores, while the seeds can be used to treat cracking feet. According to Kokwaro (1993), mature leaves can be picked and pounded in a mortar or a grinding stone, the material transferred to a leaf of smooth nature, a little water is added and the leaf folded so as to provide a crack at one of the two points. A drop of this preparation is put into itchy eye in treatment of trachoma especially in children (Kokwaro, 1993). The root decoction is also used to treat syphilis, hookworm, and stomach ache. Bark decoction used for diarrhea and venereal diseases. Root infusion used for impotence or sterility. Pounded leaf juice used as eye drop for conjunctivitis in cattle (Kokwaro, 1993). *Ximenia americana* has also been reported to treat throat infection, malaria and dysmenorrheal and has also been used for wound healing in Mali (Diallo *et al.,* 2002; Gronhaug *et al.,* 2008).

In Nigeria the tree has been used against malaria, leprotic ulcers and skin diseases, it is also used against schistosomiasis, fever, diarrhoea, ringworm; craw-craw and tooth ache (Ogunleye and Ibitoye, 2003; Burkill, 1997). The chemical composition of *X. americana*

has scarcely been investigated. However, Sambunigrin was isolated from the leaves in 1938 (Finnemore and Cooper, 1938).

Over the recent past, fatty acids, volatile oil constituents, sesquiterpenes, triterpenes and phytosterols have been reported from the seeds, leaves and stems of *Ximenia americana* (Eromosele and Eromosele, 2002; Mevy *et al.,* 2006; De Araujo *et al.,* 2008, 2009). However the chemistry of the roots is still unknown. Pharmacological studies of alcoholic extracts have shown antimicrobial (Ogunleye and Ibitoye, 2003; Omer and Elnima, 2003; Kone *et al.*, 2004), antiviral (Asres *et al.*, 2001), antioxidant and anti-parasitic (Maikai *et al.,* 2010) and analgesic activities (Hemamalini *et al.,* 2011). Water extracts have shown complement fixing ability (Diallo *et al.,* 2002), and anticancer activity (Voss *et al.,* 2006).

Figure 2.4: *Ximenia americana* plant (Source: Author , 2013)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The plants were collected from three counties namely Embu, Kajiado and Baringo Counties.

3.1.1 Embu County

Embu County is bordered by Kitui County to the East, Kirinyaga to the West and Tharaka Nithi to the North. The County lies approximately between UTM Zone 37^0N (0291326, 0387864 Eastings and 9989543, 9986270 Northings). It occupies a total area of 708 Km^2 (Republic of Kenya, 1982). The precipitation pattern in Embu County is bi-modal with two distinct rainy seasons. Long rains occur between March and June while the short rains fall between October and December. The County receives an average of about 1206mm of precipitation annually. It lies on an alttitude of about 1350 metres above the sea level close to the foot hill of Mt. Kenya. Temperatures range from a minimum of 12 °C in July to a maximum of 30 °C in March with a mean of 21 °C. The soils in the area have high clay and about 2.0% carbon content. The texture of the soils is moderate and has the capacity to hold water (Sombroek *et al.,* 1982).

3.1.2 Baringo County

Baringo County is bordered by Turkana County and West Pokot County to the North, Samburu County and Laikipia County to the East, Nakuru County and Kericho County to

the South, Uasin Gishu County to the South West and Elgeyo Marakwet County to the West. It covers an area of 8,655 square kilometres. Baringo County lies between Latitudes 00 degrees 13" South and 1 degree 40" north and Longitudes 35 degrees 36" and 36" degrees 30" East (Republic of Kenya, 1982). The county has two rainfall seasons with an average of about 1000mm in the highlands and 600mm in the lowlands. The highlands regions are mainly dominated by alluvial soils which are suitable for agriculture (Sombroek *et al.,* 1982). The altitude is approximately 1000m to 2600m above the sea level and this contributes to the weather patterns. The temperatures vary between 25 to 30 degrees Celsius, with a rise to an average of 35 degrees Celsius in January (Bille and Heemstra, 1979).

3.1.3 Kajiado County

Kajiado County borders Nairobi and extends to the Tanzania border further south. It occupies total area of 21,292.7 km^2. The landscape of Kajiado County consists of plains and volcanic hills and valleys (Republic of Kenya, 1982). The County is semi arid and receives low amounts of rainfall annually of approximately between 500 and 1250 millimeters per annum. The rainfall which is unpredictable is at times as low as 250 millimetres. The County has two rain seasons with long rains received in March and May and the short rains are received in October and December (Bille and Heemstra, 1979). The soils are not favourable for growth of most of the crops due to the poor texture which in turn translates to low water holding capacity and high water infiltration rates (Touber, 1983). The soils are very susceptible to erosion processes such as wind

erosion due to their light weight nature. It has scarce vegetation dominated by shrubs and herbs (Pratt, 1977).

3.2 Collection sites and processing of plants

Plants were selected for the study based on their ethno medical and literature information. Specifically, they were selected on the basis of their traditional reputation to treat fevers or malaria locally. The specimen were identified and authenticated by a taxonomist and voucher specimens deposited at the East African Herbarium, National Museum of Kenya. The different plant parts were air-dried at room temperature under shade to a moisture content of 13% and pulverized individually using a laboratory mill (Christy & Norris Ltd., Chelmsford, England) at the Center for Traditional Medicine and Drug research, KEMRI. The powders were packed in air tight polythene bags, labeled and stored in the dark until used.

3.3 Study site

The final study was conducted at Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. The study was based at the Center for Traditional medicine and drug research. The mandate of the Center for traditional Medicine and Drug Research is to document herbal remedies used by various herbalists and Traditional Herbal Practitioners (THPs) to treat the diseases. It is also involved in evaluation of safety, efficacy and chemistry of medicinal plant preparations claimed to treat diseases. The *in vitro* assay was carried out in the Malaria Culture Laboratory. The *in vivo* assay was carried out at the animal house which is a facility specifically designed for rearing of experimental mice. The approval to undertake the study was given by KEMRI institution's Scientific Steering Committee and

the Ethical Review Committee. The KEMRI animal care and use committee (ACUC) gave approval for the animal use (Appendix 17).

Table 3.1: Plants collected, voucher numbers, parts collected and place of collection

Botanical Name	Family	Local name	Voucher number	Part used	County collected	Date collected
Fuerstia africana.	Lamiaceae	Ooito-doraik	EK 744	AP	Kajiado	12/07/11
Sericocomopsis hildebrandtii	Amaranthacea	Oloitoruj-ilpeles	EK 743	AP	Embu	06/09/11
				R	Embu	
Pentas lanceolata	Rubiaceae	Olkilaki-olker	EK 739	AP	Kajiado	27/10/11
Ximenia americana	Olacaceae	Muiyeng wet	EK 711	SB	Baringo	25/06/11

3.3 Extraction using methanol

Methanol soluble compounds from the powdered plant material was extracted by soaking in 500 ml of methanol for 48 hours at room temperature and filtered with filter paper (whatman No.1). The plant material was again re-extracted immediately after filtration with 300ml of methanol for 12 hours and the filtrates pooled and concentrated under vacuo using a rotatory evaporator (BUCHI Rotavapor R-124) at 40^0C until dry

(Harborne, 1998). The concentrate was transferred to an air tight bijou bottle, weighed, labeled and stored at 4^0C until used.

3.4 Extraction using water

Aqueous compounds from the powdered plant material (50 g) was extracted by soaking it in 500 ml of distilled water in a water bath at 60°C for 1 hour, filtered with filter paper (whatman No.1) and freeze dried with a freeze drier (Edwards freeze dryer Modulyo). The freeze dried powder was weighed, labeled and stored in an air tight bijou bottle at 4 °C until used.

3.5 Formulations of plant extracts and chloroquine for *in vitro* and *in vivo* bioassays

3.5.1 Methanol extracts

Methanol extracts were not readily soluble in water hence 10mg of the exract was first dissolved in 1ml of dimethyl sulfoxide (DMSO, Sigma chemical CO., St. Louis, MO, USA) followed by subsequent dilution to lower concentration of DMSO, to <1% to avoid carry over (solvent) effect (Elueze *et al.*, 1996). The first row had a final concentration of100 µg/ml. For the *in vivo* assay 0.20g of extract was weighed and dissolved in 5mls of 10% tween 80 to give a final concentration of 500mg/kg/day.

3.5.2 Aqueous extracts

For the *in vitro* experiments, 0.05 g of the extract was dissolved to a final volume of 5ml (stock solution of10,000 µg/ml). Each of the extracts was then filter sterilized with syringe adaptable 0.22 µm filters into sterile Bijoux bottles in the laminar flow hood (Bello Glasses Inc., USA) and stored at -20ºC. This was later diluted using distilled

water to make 1,000µg/ml. The final volume in each well was 250 µl and this gave the first row a concentration of100 µg/ml. In the *in vivo* assay, 0.20g were weighed and dissolved in 5mls of distilled water. This gave a final cconcentration of 500mg/kg/day for each mouse.

3.5.3 Preparation of Chloroquine and Artemisinine

Chloroquine diphosphate and Artemisinine were prepared so that the plate's first row concentration was 1µg/ml, sterilized and stored at -20^{0}C. The drugs were sterilized using 0.22 syringe adaptable filters in the laminar floor hood (Bello Glasses Inc, U.S.A).

3.6 Source and maintenance of malaria parasites

3.6.1 Source and cryopreservation of the human malaria parasites

To test the antiplasmodial activities of the aqueous and methanol plant extracts *in vitro*, the CQ susceptible (D6) and CQ resistant (W2) clones donated by the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington DC were used. The method of Rowe *et al.,* (1968) was adopted for cryopreservation of the parasites to ensure enough supply of laboratory-adapted isolates as well as having manageable culture flasks. Thick smear was made to ascertain that the cultures for cryopreservation were not contaminated. The culture was transferred into 15 ml centrifuge tube and centrifuged at 1500 r.p.m for 5 minutes at 20ºC. After aspirating the supernatant, packed cell volume (PCV) was estimated and an equal volume of Rowe's cryosolution was added. Aliquots of 0.25 ml were then put into 2 ml cryovials (NUNC®, USA) placed in aluminum canes, and preserved in liquid nitrogen.

3.6.2 Source and maintenance of rodent malaria parasite

To test for antimalarial activities of the aqueous and methanol extracts, the mouse-infective, CQ sensitive *P. berghei* strain ANKA donated by International Livestock Research Institute, ILRI, Kenya was used. The parasite was inoculated intraperitoneally (i.p.) into a male outbred swiss albino mouse, the donor mouse to the experimental mice. Six days after parasite inoculation, its parasitaemia was assessed microscopically by examining Giemsa stained thin blood films from the tail vein, the mouse was bled under anaesthesia by cardiac puncture into a heparinized tube and its erythrocyte density determined using a haemocytometer. The parasitaemia was adjusted downwards using physiological saline and each of the recipient male swiss albino mouse, weighing 20 ± 2 g was inoculated i.p. with approximately 10^7 parasitisized erythrocytes in volumes of 0.2 ml (Ishih *et al.,* 2003). The inoculated mice were randomized into groups of 5 per cage and maintained in the animal facility on a commercial diet and water, *ad libitum*.

3.7 Source and preparation of human host cells and serum

3.7.1 Human host cells

Uninfected human blood group O+ erythrocytes (<28 days old) served as host cells. Briefly, 60 mls of blood from every recruited volunteer who had not contracted malaria or visited a malaria endemic area in the past two months was drawn into 15% v/v acid-citrate-dextrose (ACD). By use of a questionnaire and consent form, it was ascertained that the donor had not taken any antimalalrial or antibiotic drugs. Uninfected blood was then centrifuged at 3600 r.p.m for 10 minutes at 4 ºC to get rid of plasma and white blood

cells. The plasma and buffy coat at the top of the cell pellet was aspirated and discarded. The red pellet was washed twice with two volumes of wash medium and centrifuged again. After the last wash, the packed cells were resuspended in an equal volume of the wash medium to obtain 50% hematocrit then exposed to a gas mixture of 92% N_2, 5%CO_2 and 3%O_2, stored at 4 ºC and used within 2 weeks.

3.7.2 Human serum

Consent for one unit of blood from at least three adult volunteers with blood groups A+ and O+ who have tested negative for HIV and Hepatitis and have not contracted malaria or visited malaria endemic area in the past two months was obtained. Blood from each volunteer (500 ml) was drawn aseptically into blood bags (Fenwell 4R0001) without anti-coagulants separately. The blood was allowed to clot at room temperature for 90 minutes followed by overnight storage at 4ºC. Serum was carefully dispensed into sterile 50ml centrifuge tubes the next day and centrifuged at 3000 r.p.m for 10 minutes at 4 ºC to separate contaminating cells. The serum was then transferred into 75cm^2 (250 ml) sterile conical flasks and heat inactivated in a water bath at 56 ºC for 40 minutes. They were then aliquoted into sterile 10ml snap-top tubes and placed in upright position at -20 ºC overnight and then kept at -70 ºC until used. Using each batch of serum separately, a small amount (50 ml) of complete medium with serum (CMS) was prepared and used to maintain a culture for one week to test whether each batch supported growth.

3.8 Preparation of reagents

3.8.1 Preparation of wash media material

Wash medium (WM) was prepared by mixing 95.8% v/v of RPMI 1640, 4.2%v/v HEPES medium and 5% w/v sodium carbonate as described by Rowe *et al*.,(1968). The Wash medium was flushed with 92% N_2, 5% CO_2 and 3% O_2, sealed, kept at 4 ºC and used within 14 days.

3.8.2 Preparation of complete Culture Medium with Serum (CMS) material

The culture medium consisted of RPMI 1640 (10.4g/l) powdered medium without *p*-aminobenzoic acid (PABA) and lactic acid dissolved in 960ml of distilled autoclaved water. This was supplemented with 10% human serum, 25 Mm (5.9g/l) *N*-2-hydroxyethylpiperazine-N-2-ethanesulfonic acids (HEPES) and 25 Mm $NaHCO_3$ as described by Schlichtherle *et al*., (2000). The media was filter sterilized using a vacuum pump and 0.22 µm filters and stored at 4 ºC for not longer than 4 weeks.

3.8.3 Preparation of 5% sodium bicarbonate material

Sodium bicarbonate was prepared by dissolving 2.5 gm. $NaHCO_3$ in 50 ml distilled water and filtered through 0.22 µm syringe adaptable filter then stored at 4 ºC for not longer than 2 weeks.

3.8.4 Preparation of Acid-citrate-dextrose (ACD) material

Acid-citrate-dextrose (ACD) was used as an anticoagulant while collecting RBC. It was prepared using 22.0 g tri-sodium citrate, 8.0 g citric acid and 24.4 g dextrose then dissolved in 1 litre sterile water. The ACD was then re-sterilized by filtration through 0.22 µm filter and stored at 4ºC until use.

3.9 Study design

Experimental design was used. Where plant extracts were tested for their antiplasmodial activity *in vitro* and antimalarial potential in mice. Reference on the efficacy of the extracts was based on positive controls. The *in vitro* positive controls consisted of Artemisinine and Chloroquine. *In vivo* controls were composed of a positive control using Chloroquine which is considered as a standard drug for this strain and a negative control which consisted of distilled water for water extracts and tween 80 for methanol extracts which acted as the vehicles. A cage of five mice was used for each plant extract tested and the controls. The experiments were done in triplicates.

3.10 *In vitro* antiplasmodial assay

Methanol and water extracts of the 4 selected medicinal plants were tested against a chloroquine sensitive *P. falciparum* D6 strain. Several steps were involved in this assay; they are described briefly below.

3.10.1 Thawing of cryopreserved parasites

The method of Rowe *et al.,* (1968) was adopted for this work. The Sierra Leonean CQ-sensitive, D6 strain and the CQ resistant W2 strain of *P. falciparum* was used in the study. It was cryopreserved in liquid nitrogen in a cold room maintained at -80°C. The specimens were identified from the logbook. The vials containing the parasites were removed, thawed in a water bath at 37°C and aseptically transferred to 15 ml centrifuge tube, in a laminar flow hood. They were centrifuged at 1500 rotations per minute (r.p.m) for 5 minutes at 20ºC. The supernatant was aspirated (using a sterile unplugged Pasteur

pipette) and 0.3 ml of 3.5% NaCl (w/v) added, mixed well and centrifuged again at 1500 r.p.m. for 5 minutes and 20°C. The supernatant was removed and 50% RBC and Culture Medium with Serum (CMS) was added.

3.10.2 Maintaining revived cultures

Parasite cultivation was carried out aseptically in a laminar flow hood based on the *in vitro* technique described by Trager and Jensen, (1976). Briefly, cultures were maintained in volumes of 5 ml, 6% hematocrit and incubated at 37ºC with daily changes of the culture medium and gassing (5% O_2, 3% CO_2 and 92% N_2). Slides were usually prepared after 48 hours to assess the status of the culture by applying a small drop of the cell suspension after aspirating the medium onto the glass slide. Thin smears were fixed with absolute methanol, allowed to dry, and stained for 10 minutes with 10% Giesma stain. The slides were gently washed with water, dried and examined under a microscope (100x oil immersion).

Dilution or sub-culturing (preparing new cultures from old cultures) was done when the percentage parasitaemia was high, and when no contamination was found on examining the slide under the microscope. The necessary volumes of culture medium, 50% fresh erythrocytes and medium needed for 5 ml, 6% hematocrit culture were calculated from the formulae:

$$\text{Culture volume (CV)} = \frac{5}{D}$$

$$\text{50\% erythrocytes (EV)} = \frac{6}{50 - CV}$$

$$\text{Medium Volume} = 5 - (CV + EV)$$

Where, D is the reciprocal of the desired dilution factor (for example, D= 10 for 1:10 dilution). The appropriate volume of 50% RBC and medium were mixed together in new 25 cm^2 (50 ml) flasks using sterile technique, gassed (3% CO_2, 5% O_2 and 92% N_2) and incubated (37°C) for 20 minutes. The desired volume of old culture was then added gassed and incubated at 37^0C.

3.10.3 *In vitro* drug sensitivity test

The semi-automated micro dilution technique described by Desjardins *et al.,* (1979) for assessing *in vitro* antimalarial activity and later modified by Le Bras and Deloron, (1983) was adopted in the drug sensitivity studies for the plant extracts, chloroquine and artemisinine against *P. falciparum* isolates. In brief, the 96 flat bottomed well plates (a matrix of 8 rows and 12 columns) were set such that all wells except for control wells contained 25 µl of doubling concentrations of drug and extract solutions. Parasitized red blood cells (200 µl) were added so that the total volume per well was 225 µl.

3.10.4 Preparation of test dilutions in microtitre plates

The 96 well flat-bottomed micro-titre plates (NUNC®, USA) with covers were used for drug sensitivity tests. Under sterile conditions in the laminar flow hood (Bellco Glass Inc., USA), the plates were laid along the columns (1-12). Aliquots (25µL) of culture media was added with a multi-channel micropipette from row B to H, exempting row A wells. The extracts and drugs (50 µl) were added in duplicate into wells of row A, (this meant that each extract held two columns in one plate therefore accommodated 6 drugs/extracts in duplicate). Two-fold dilutions were done by transferring 25 ul of the drug/extract with a multi-channel micropipette from row A down to row G (last 25 µl from G wells discarded). Row H wells were exempted since they served as controls (wells without drugs/extracts). Thus, row A wells had a concentration of 100 µg/ml, B wells 50 µg/ml, C wells 25 µg/ml, D wells 12.5 µg/ml, E wells 6.25 µg/ml, F wells 3.125 µg/ml and G wells 1.5625 µg/ml wells. Consequently row A had 100% drug concentration while those of G only 1.5625% of the test sample. The plates were then covered and kept at 4ºC.

3.10.5 Addition of parasites to the pre-dosed plates

The test culture which was at ring stage, with percentage parasitaemia (% P) of 4% and growth rate (GR) of 3% was used during sensitivity tests for calculation of % P and GR. After examining the parasites under the microscope, the % P of the test culture to be added to the wells of predosed plates was adjusted to 0.4% and hematocrit (hct) adjusted to 1.5% with 50% RBC. Culture Medium with Serum (CMS) was put into a 25 cm^2 (50 ml) culture flask and the appropriate volume of 50% RBC added. This was flushed with a

gas mixture of 3% CO_2, 5% O_2 and 92% N_2 (BOC®, Kenya) and placed in a 37ºC incubator for 5 minutes. Using sterile technique, in the laminar flow hood, the appropriate volume of test culture was added into the flask containing CMS and 50% RBC, and gently swirled in a circular motion to mix. Meanwhile the previously predosed plates were warmed at 37ºC in the incubator for 20 minutes. The plates were then retrieved and placed in the laminar flow hood and the test culture put into sterile tissue culture dishes (Lux®, USA). Using 1-200 µl tips (Fisherbrand ®, USA) and a multi-channel pipette, aliquots of 200 µl of the mixture were dispensed into the wells except for H_9 to H_{12} (4 wells). To these, unparasitisized red blood cells (UPRBC) mixed with CMS at the appropriate concentration were added. This served as the negative control while H_1-H_8 served as the positive control (parasitized red blood cells with no drug).

3.10.6 Incubation of the plates

After replacing the lids of microtitre plates and shaking the plates gently, they were placed into the gas tight box, which had a damp cotton gauze to maintain a humid atmosphere in the chamber. The gas box lid was replaced and the airtight box flushed with 3% CO_2, 5% O_2 and 92% N_2 (BOC®, Kenya) and incubated at 37ºC. After 48 hours, [G-^{3}H] hypoxanthine (0.5 µCi/well) was pulsed in aliquots of 25 µl into each well and the plates incubated for a further 18 hours. Then freezed for 24 hours before harvesting.

3.10.7 Harvesting of cells and scintillation counting

The cells were harvested using an automatic 96 well cell harvester onto glass fibre filter mats for each row from A to H. The filters were then dried at 37ºC for five minutes and then placed into a plastic sample bag and 5 ml liquid scintillation fluid added. The open end of the sample bag was heat-sealed; placed into a cassette and read using a scintillation β counter (Microbeta counter). Disintegrations per minute were calculated for each sample. The counts per minute (cpm) for each sample represented the incorporation of [G-^{3}H] hypoxanthine into the parasite nucleic acids.

3.10.8 Determination of inhibitory concentration (IC_{50})

In vitro results are given as the inhibition concentration (IC_{50}). This refers to the drug concentration capable of inhibiting growth *P. falciparum* by 50% and is obtained by logarithmic transformation of both concentration and counts per minute (cpm) (Sixsmith *et al*., 1984) using the formula:

$$IC_{50}=\text{antilog}\left(\log X_1+\left[\frac{(\log Y_{50}-\log Y_1)(\log X_2-\log X_I)}{(\text{Log } Y_2-\log Y_1)}\right]\right.$$

Where Y_{50} is the cpm value midway between the average of parasitized control (parasite present, on drug) and X_1, Y_I, X_2 andY_2 are the concentrations and cpm values for the data points above and below the cpm midpoints. Antiplasmodial activity was classified as follows: High at $IC_{50} \leq 10\mu g/ml$, moderate at 10-50µg/ml, low at ≥50 following the protocol described by Gathirwa *et al*., (2007).

3.11 *In vivo* antimalarial assay

The assay protocol was based on a 4-day suppressive test using the rodent malaria parasite *P. berghei* as described by Peters *et al.*, (1975). Female Swiss albino mice (weighing 20 ± 2 g) were used for this assay. The mice were bred in standard macrolon type II cages in air-conditioned rooms at 22^{o}C, 50–70% relative humidity, fed on commercial rodent food and given water *ad libitum.* Each cage consisted of five mice. The reference drug, chloroquine diphoshate was administered orally at a dose of 5 mg/kg body weight to the positive control group of mice.

3.11.1 Infection of mice

Each mouse was inoculated intraperitoneally with 1x10^{7} erythrocytes (red blood cells, RBC) parasitized with *P. berghei* ANKA strain, in a saline suspension of 0.2 ml on day 0. The randomly infected mice were then divided into groups of 5 which consisted of a negative control that received placebo 10% tween 80, a positive control which received 5mg/kg body weight of chloroquine diphoshate and the rest consisted of the plant extracts at 500mg/kg body weight.

3.11.2 Treatment and evaluation of the extracts on blood schizonticidal activity

The mice were treated daily from day 0 (immediately after infection) to day 3, through oral route with an extract dose of 500 mg/kg/day as described by Gessler *et al.*, (1995). The mice in the positive control group were given chloroquine diphosphate (dissolved in

10% Tween 80) at 5 mg/kg/day orally. Those in the negative control group received the placebo (10% tween 80) at 0.2 ml/kg/day.

3.11.3 Parasitaemia determination

Parasitaemia in each group of mice was assessed on day 4 (96 hours post infection) using Giemsa-stained thin films made from tail blood. Percentage chemosuppression (parasite reduction) of each dose of extract was calculated by the formula proposed by Tona *et al.*, (2001).

(**A**-**B**)/**A**) x 100

Where **A**-is the mean parasitaemia in the negative control group.

B-is the parasitaemia in the test group.

The mean survival time (days) for all groups of mice were determined and at the end of each experiment the mice were euthanized in CO_2 chamber and incinerated. The standard deviations for the mean values were calculated as described by Armitage and Berry, (1991). Suppression of parasitaemia (Chemo-suppression) in mice and mean survival time of the animals were used as measures of drug efficacy. Test samples were categorized as highly active when chemo suppression was above 70%, moderately active between 50 and 69%, lowly active between 30 and 49%, inactive below 30% as categorized earlier by Muregi *et al.*, (2007), Carvalho *et al.*, (1991), Andrade - Neto *et al.*,(2003) and Gathirwa *et al*., (2011).

3.12 *In vitro* cytotoxicity assay

In vitro cytotoxicity assay was carried out following a modified rapid colourimetric assay described by Mosmann, (1983) using actively dividing sub-confluent Vero E 99 cells. Briefly, the Vero cells were maintained in Eagle's Minimum Essential Medium (MEM) containing 10 % fetal bovine serum (FBS), in T-75 cell culture flasks incubated at 37ºC in 5% CO_2. Upon attainment of confluence, cells were detached by trypsinization and pooled in a 50ml tube. The cells were then resuspended in 40ml fresh MEM. The number of viable cells was determined by a tryphan blue exclusion test. An aemocytometer was used to aid in counting viable cells under 40x objective lens of an inverted microscope. 100 µl of the cell suspension at 2 x 10^5 cells per ml was seeded into each well of a 96-well plate and incubated at 37ºC in 5% CO_2 for 24 hrs to attach. The test sample extracts diluted with MEM to a starting concentration of 100 µg/ml were seeded in duplicate in columns in a 96 well plate while the third column was left blank as the control; Columns 1, 2, 4, 5, 7, 8 and 10, 11 had drug extracts and cells whereas columns 3, 6, 9 and 12 that served as controls contained drug extracts but no cells.

Row H had the highest drug concentration and serial dilution of 50 µl was carried out upwards from row H to row B, (row A had no drug extracts). Chloroquine (CQ) drug with an Initial concentration of 10 µg/ml in 1% DMSO was used as the positive control standard for the experiment. The plates were then incubated for 48 hours at 37^{o}C in a 5 % CO_2 incubator. At the end of the incubation time, 10 µl of MTT dye was added into each well and cells were incubated for another 4 hours, (with 0.8 mg/ml of MTT), dissolved in Phosphate buffered saline (PBS).

After 4 hours of incubation with MTT, the cells were observed for dye intake, after which all media was removed from the plates and 100 µl of DMSO added into each well. The plates were then read on a scanning multiwell spectrophotometer (Multiskan Ex labs systems) at 562 nm and 620 nm as reference.

Data was analysed as follows:

$$\% \text{ Cell viability } (CC_{50}) = \left(\frac{[OD_{\text{sample } 562} - OD_{620}]}{[OD_{\text{control } 562} - OD_{620}]} \right) \times 100$$

Where OD = optical density

Data was transferred on to a graphic programme (EXCEL) and expressed as percentage of the untreated controls. Concentration required to cause visible alterations in 50% of intact cells (CC_{50}) was determined. Cytotoxicity was classified as: Cytotoxic at CC_{50} < 2µg/ml, moderately cytotoxic at CC_{50} between 2 - 89µg/ml, not cytotoxic at CC_{50} > 90 µg/ml as categorized by Rukunga and Simons, (2006). CC_{50} is the concentration or dose of extract that kills 50% of the cells.

3.13 *In vivo* acute toxicity

In vivo acute toxicity was determined in mice using slightly modified standard procedures (Lorke, 1983). Female Swiss albino mice with a mean body weight of 20±2 g bred at KEMRI, Nairobi, Kenya were used for the study. They were randomly divided

into groups of 5 mice, one group for each extract, plus one negative control group that was given the vehicle tween 80. Each mouse was starved overnight prior to treatment. Increasing doses of the test extract (500,889,1581,2811,5000 mg/kg/day) was administered orally, 30 minutes later the mice were given water, then food after another 30 minutes. Each mouse was checked for signs of toxicity within 1-8 hours, at 24 hours and thereafter daily for 14 days. They were weighed on day 0, 3, 7 and 14. At the end of each experiment the mice were euthanized in CO_2 chamber and incinerated. The number of deaths per group after 24 hours was used to calculate the lethal dose for 50% of the experimental mice (LD_{50}), by plotting the percent mortality against the concentration of doses administered. The value of the LD_{50} was extrapolated from the graph.

3.14 Data management and analysis

Statistical analysis was done using StatView Version 5.0. Analytical tool used was student t-test. It was used to determine significant difference between quantities of extracts obtained between methanol and water extraction techniques and the significance difference between the IC_{50} values obtained from the plant extracts and the controls. The student t-test was also used to determine the significance difference between the parasite density of mice receiving the different plant extracts and the positive control, and also the significance of mean survival time of mice receiving the plant extracts compared to the controls; chloroquine and 10% Tween 80. All analysis were done at 95% confidence interval/level, hence significance was determined at $p<0.05$ or >0.05 where $p\leq0.05$ was considered as significant.

In the *in vivo* antimalarial bioassays extracts with a chemo suppression of >30% were considered active following Muriithi *et al.*, (2002) and those which prolonged the mean survival time greater than infected untreated controls were also regarded as active. For acute toxicity, a non toxic dose of 5000 mg/kg body weight or more was considered safe for that extract. The highest examinable oral dose for any extract was 5 g (5000 mg). Generally, if an extract showed no toxicity at that dose, it was considered very safe and was not examined further.

CHAPTER FOUR

RESULTS

4.1 Yields of water and methanol extracts

The plant species and the yields of water and methanol extracts individually extracted, each from 50g of the powdered plant material is shown in Table 4.1. For all the plants, the yields of the water exracts were higher compared to the yields of the methanol extracts. The difference in the yields was statistically significant (P=0.023).

Table 4.1: Plant species and yield of water and methanol extracts

Botanical name	**Voucher specimen number**	**Family**	**Part**	Dry weight of extract %y **Water* extract**	**Methanol* extract**	**P values**
F. africana	EK 744	Lamiaceae	Aerial part	9.4	3.73	0.000
S. hilderbrandtii	EK 743	Amaranthacea	Aerial part	7.04	2.08	0.009
			Roots	10.58	2.32	0.000
P. lanceolata	EK 739	Rubiaceae	Aerial part	7.06	2.53	0.001
X. americana L	EK 711	Olacaceae	Stem bark	16.38	11.62	0.002

*= Value statistically significant P≤0.05 (Student t test: mean of water extract yields versus means of methanol extract yields).

4.2 *In vitro* antiplasmodial assay

Results of *in vitro* antiplasmodial assay of methanol and aqueous extract of the four selected medicinal plants against *Plasmodium falciparum* D6 and W2 strains are depicted in Tables 4.2 and 4.3 respectively. Methanol extracts potrayed high invitro activity against D6 strain. Aerial parts of *F. africana* (methanol extract) was the most active against D6 strain of *P. falciparum* with an IC_{50} of 1.841 µg/ml, followed closely by stem bark of *X. americana*, methanol extract with IC_{50} of 2.108 µg/ml.

Table 4.2: *In vitro* antiplasmodial activity (IC_{50}) of aqueous and methanol extracts of selected medicinal plants and standard drugs against D6 strain of *Plasmodium falciparum*

				P values
Plant	**Part**	**Aqueous extract*** IC_{50} ± SD (µg/mL)	**Methanol extract*** IC_{50} ± SD (µg/mL)	
S. hilderbrandtii	AP	18.004±3.62	3.153± 1.24	0.009
	R	4.096±1.69	2.12± 0.56	0.09
F. Africana	AP	17.035± 4.31	1.841± 0.82	0.019
P. lanceolata	AP	7.127± 1.73	3.744± 0.42	0.047
X. Americana	SB	9.621±2.45	2.108± 0.27	0.027
Chloroquine	-	7.649± 1.82		
Artemisinine	-	0.962± 0.33		

IC_{50}= Concentration that killed/inhibited 50% of parasites/ relative to negative control, S.D. = standard deviation, the drugs were tested in triplicate, Standard drugs were chloroquine and artemisinine, AP =aerial part, SB = stem bark, R = root

*****= value statistically significant, $P \leq 0.05$ (Student t test: IC_{50} values of water extracts verses IC_{50} values of methanol extracts)

Aerial parts and roots of *S. hilderbrandtii* methanol extracts also exhibited high antiplasmodialactivity with IC_{50} value of 3.153µg/ml and 2.12µg/ml respectively. The high *in vitro* activity was still observed for aerial parts of *P. lanceolata* methanol extract which had an IC_{50} of 3.744µg/ml against D6 strain of *P. falciparum.*

Aqueous extracts of *S. hilderbrandtii*, roots, aerial parts of *P. lanceolata* and stem bark of *X. americana* had high activity (IC_{50}, 4.046µg/ml , 7.127 µg/ml and 9.621) respectively against D6 strain. Aerial parts of *F. africana* and *S. hilderbrandtii*, had moderate activity (IC_{50}, 17.035 µg/ml, and 18.004 µg/ml) respectively.

Table 4.3: *In vitro* antiplasmodial activity (IC_{50}) of aqueous and methanol extracts of selected medicinal plants and standard drugs against W2 strain of *Plasmodium falciparum*

	Part	**Aqueous extract** * IC_{50} ± SD (µg/mL)	**Methanol* extract** IC_{50} ± SD (µg/mL)	**P values**
S. hilderbrandtii	AP	78.695 ±0.7	12.688±4.69	0.000
	R	54.166 ±4.71	14.851±3.37	
F. africana	AP	37.981±2.43	6.227± 0.12	0.002
P. lanceolata	AP	43.151±6.32	14.903±2.14	0.013
X. americana	SB	83.489 ±7.21	36.791±4.51	0.001
Chloroquine	-	56.117±0.22 µg/ml		
Artemisinine	-	3.56±0.17 µg/ml		

IC_{50}= Concentration that killed/inhibited 50% of parasites/ relative to negative control, S.D. = standard deviation, the drugs were tested in triplicate, Standards drugs were chloroquine and artemisinine, AP =aerial part, SB = stem bark, R = root

*= value statistically significant, $P \leq 0.05$ (Student t test: IC_{50} values of water extracts verses IC_{50} values of methanol extracts)

With the W2 strain aerial parts of of *F. africana* methanol extract, had the highest activity (IC_{50}, 6.227µg/ml), while aerial parts and roots of *S. hilderbrandtii*, aerial parts of *P. lanceolata* and the stem bark of *X. americana* (methanol extracts) had moderate activity (IC_{50}, 12.688µg/ml, 14.851µg/ml, 14.903µg/ml, and 36.791 µg/ml), respectively.

Water extracts on the other had relatively lower activities in comparison with their methanol counterparts against W2 strain of *P. falciparum*. Aerial parts of *F. africana* and *P. lanceolata* had moderate antiplasmodial activities with IC_{50} values of 37.981µg/ml and 43.151µg/ml respectively against W2 strain of *P. falciparum.* Generally, for both strains of the parasite, artemisinine perfomed significantly ($P<0.05$) better than chloroquine (Table 4.2 and 4.3). Notably D6 strain was more sensitive to the plant extracts and the conventional drugs compared to W2 as indicated by the significantly higher values of IC_{50} observed in W2 tests than D6 strain ($P<0.05$).

4.3 *In vivo* assay

Results of *In vivo* anti-malarial assay of the plant extracts against *Plasmodium berghei* in mice are summarized in Table 4.4. Seven extracts were classified as active with chemosuppression ranging from 30.29% to 64.92% while three were classified as in active with a chemosuppressions of 12.63%, 17.53%, and 27.88%. Aerial parts of *P. lanceolata* (methanol extract) and stem bark of *X. americana* (water extract) were the most active with chemosuppression of 64.92% and 54.87% respectively. There was significant parasite density reduction ($P=0.012$) in animals treated with the active plant extracts/conventional drugs compared to the ones treated with the negative control of

10% tween 80 (control) (Table 4.4). The survival time of mice that received medicinal plant extracts/conventional drugs exhibiting high chemosuppression lived slightly longer than those that received 10% tween 80 although this difference was statistically insignificant (P=0.08).

All the water extracts were active against *P. berghei* in mice with Chemosuppresion of 30.80%, 33.89%, 41.04%, 54.87%, 30.29% for *F. africana (aerial part), S. hilderbrandtii* (aerial part), *P. lanceolata (aerial part), X .americana (stem bark),* and *S. hilderbrandtii* (root) respectively. However, only two of the methanol extracts were active. These were aerial parts of *P. lanceolata* and stem bark of *X. americana* which had Chemosupressions of 64.92% and 50.81% for respectively. Aerial parts of *F. africana*, and Aerial parts and roots of *S. hilderbrandtii,* were inactive with Chemosuppressions of 27.88%, 17.53% and 12.63% respectively.

Table 4.4: *In vivo* anti-malarial activity of selected medicinal plant extracts on *Plasmodium berghei* in mice at 500 mg/kg/day.

Extract/Drug	Part	Solvent	Parasite Density	% chemo-Suppression	Mean survival time
F. africana	AP	Methanol	25.14 ± 3.65*	27.88 ± 3.47	10.6 ± 1.51
		Water	20.79 ± 2.02*	30.80 ± 2.80	10 ± 2.94
P. lanceolata	AP	Methanol	12.23 ± 0.04*	64.92 ± 0.14	11.4 ± 3.28
		Water	20.55 ± 1.42*	41.04 ± 2.02	10 ± 2.91
	AP	Methanol	28.74 ± 0.72*	17.53 ± 2.07	10 ± 2.91
S.		Water	23.04 ± 1.88*	33.89 ± 3.41	8.8 ± 1.30
hilderbrandtii	R	Methanol	30.45 ± 2.18*	12.63 ± 1.26	9.8 ± 1.64
		Water	24.30 ± 4.29*	30.29 ± 2.31	9.2 ± 1.30
X. americana	SB	Methanol	17.15 ± 3.58*	50.81 ± 0.28	11.6 ± 3.28
		Water	15.73 ± 0.036*	54.87 ± 0.38	9.3 ± 1.25
Chloroquine (positive control)			1.11 ± 0.46	96.80 ± 1.37	15 ± 1.34
Tween 80(Negative control)			34.85 ± 2.87	-	8.8± 1.28

Results are expressed as means ± SD of 5 determinations per experiment, route of drug administration is oral, extracts tested at 500 mg/kg/day, positive control chloroquine tested at 10 mg/kg/day, negative control tween 80 tested at 0.2 ml/day.

*= Value statistically significant $p \leq 0.05$ (test verses positive control by student t-test)

4.4 Cytotoxicity Assay

Table 4.5 summarizes cytotoxicity (CC_{50}) of the plant extracts against the mammalian Vero E 99 cells. Methanol extracts of *F. africana* were moderately cytotoxic with CC_{50} of 63.45µg/ml, with a selectivity index of 34.465. The rest of the extracts were not cytotoxic at 100µg/ml; the highest concentration tested.

Table 4.5: Cytotoxicity (CC_{50}) of plant extracts to Vero E 99 cells

Extract/Drug	Part	Solvent	IC_{50} µg/ml	CC_{50} µg/ml	Selectivity index (SI)
F. africana	AP	Methanol	1.841 ±0.32	63.45±8.26	34.465
		Water	17.035±4.3	≥100	NC
P. lanceolata	AP	Methanol	3.744±0.42	≥100	NC
		Water	7.127±7.127	≥100	NC
	AP	Methanol	3.15±1.24	≥100	NC
S.		Water	18.004±3.62	≥100	NC
hilderbrandtii	R	Methanol	3.948±0.56	≥100	NC
		Water	4.046±1.69	≥100	NC
X. americana	SB	Methanol	2.108±0.27	≥100	NC
		Water	9.621±2.45	≥100	NC

SI = CC_{50} divided by IC_{50}

NC: Not cytotoxic at the highest concentration tested

AP - aerial part

R - root

SB - stem bark

4.5 Acute toxicity and effects of the extracts on body weights of Swiss albino mice

For eight of the ten extracts tested, no deaths were observed at the highest concentration tested (5000 mg/kg body weight) within 24 hours. For *X. americana*, aqueous extract 2 deaths were observed within 40 minutes. The mice treated with the rest of plant extracts continued to gain weight at a similar rate to that seen in the untreated controls and no external toxic effects or mortality were observed within 14 days (Figure 4.1 and 4.2). Stem bark of *X. americana* methanol extract was not tested since it could not dissolve at 5000mg/kg body weight the highest concentration tested.

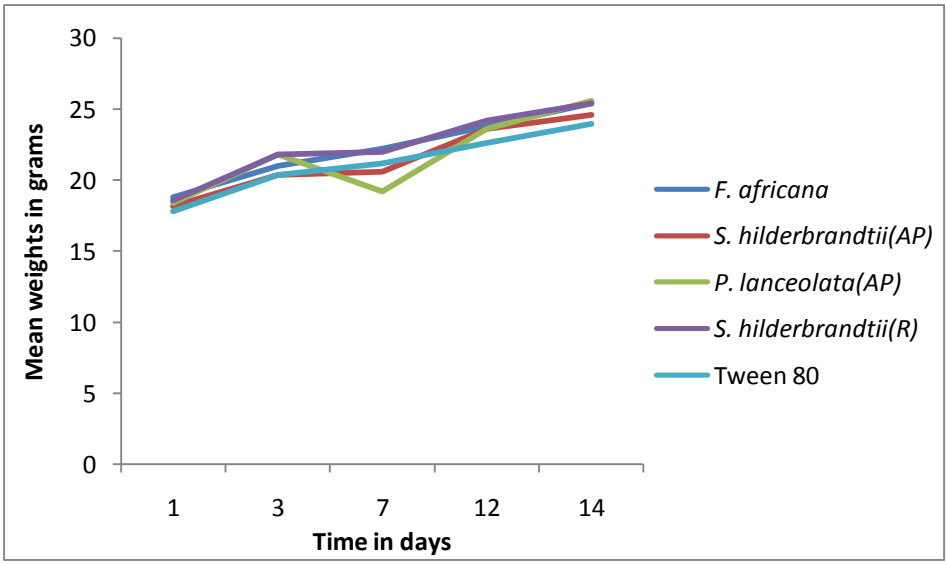

Figure 4.1: Mean weights of Swiss albino mice given methanol extracts at5000mg/kg body weight

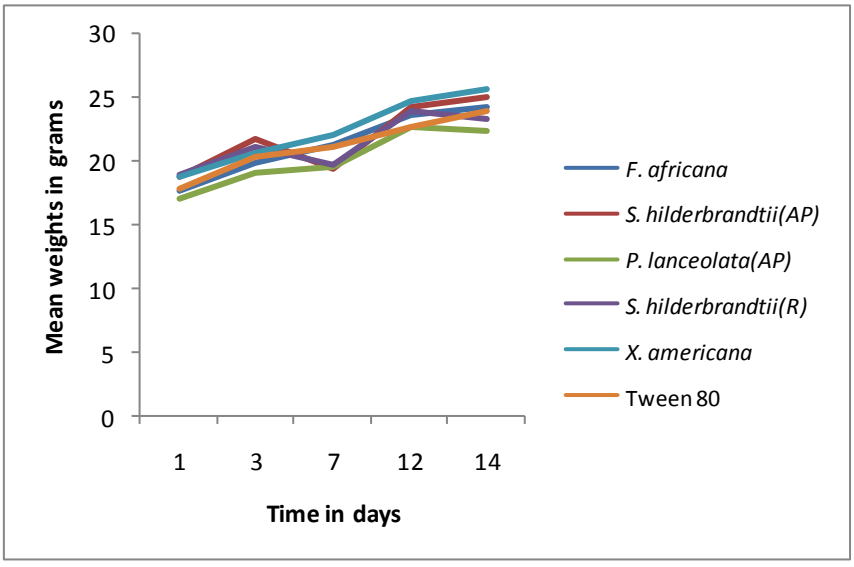

Figure 4.2: Mean weights of Swiss albino mice given water extracts at 5000mg/kg body weight

CHAPTER FIVE

DISCUSSION

The yields of water extracts were significantly (P=0.023) higher than those of methanol extracts. This could be due to polarity of the solvents, water is a more polar solvent than methanol and therefore it tends to extract more polar compounds than methanol (Muthaura *et al*. 2007). This could explain why traditional health practitioners use water as a solvent in their preparation of drugs.

The D6 strain of *P. falciparum* had significantly (P=0.004) higher activity than W2 strain. This is can be attributed to the resistant nature of the parasite strain used. Generally methanol extracts had significantly (P=0.034) higher antiplasmodial activity than water extracts against both strains of *P. falciparum*. Probably, active constituents responsible for activity extract more in methanol as already observed by Kirira *et al.,* (2006).

However, despite this relatively lower antiplasmodial activity observed in most of the water extracts, most medicinal plants are traditionally taken as aqueous extracts commonly of all plant parts (Gachathi, 1989; Kokwaro, 1993), in a cocktail with others at very high doses (Azas *et al.,* 2004) and or boiled in meat soup (that is additional fat in the meat). In some other cases extraction is done traditionally by adding honey and letting it to ferment. Fermented honey in this case introduces ethanol (Gathirwa *et al.,* 2011). Therefore traditional methods of extraction may not constitute 100% water as

solvent. In this case there is possibility of synergism between various photochemicals that against malaria parasites in humans. Also the different constituents could help extract and keep active antimalarial compounds in an aqueous solution in the traditional methods of preparation (Clarkson *et al.,* 2004). This may explain why lower activity was observed for most aqueous extracts.

Aerial parts of *F. africana* methanol extract recorded the highest activity *in vitro* against D6 strain of *P. falciparum* (IC_{50} 1.841 µg/ml) in this study. This is in agreement with literature. Previous study has reported leaves of *F. africana* to exhibit high *in vitro* antiplasmodial *activity* with IC_{50} 3.8µg/ml, $CHCl_3$ extract (Kigondu *et al.,* 2011). This was a confirmation of the activity of this plant.

The observed higher activity in this study could be attributed to plant part used. The aerial parts used in this study consisted of leaves and flowers. It is possible that flowers contained more compounds that were active against *P. falciparum* hence the higher activity observed for aerial parts of *F. africana* than the leaves used in literature. It has already been shown by Gathirwa *et al.,* (2011), that the concentration of active compounds can vary significantly between different parts of a given plant. This explains further the high activity of *F. africana* against the chloroquine resistant W2 strain of *P. falciparum* (IC_{50} 6.227 µg/ml).

Aerial parts of *P. lanceolata* had high activity *in vitro*, with IC_{50} 3.77 ±0.42 µg/ml and 7.127± 1.73µg/ml against D6 strain of *P. falciparum* for methanol and water extracts respectively. And with IC_{50} of 14.903±2.14 µg/ml and 43.151±6.32 µg/ml for methanol and water extracts respectively against W2 strain of *P. falciparum.* Previous investigation

has shown Petroleum ether extracts on roots of *P. lanceolata* to have an IC_{50} of 58.5±4.7 µg/ml against D6 strain of *P. falciparum,* while the aerial parts have been reported to have IC_{50} of 33.91±1.06µg/ml against W2 strain of *P. falciparum*. (Kigondu, 2011).

The difference in antiplasmodial activity can be attributed to the plant parts used. It is possible that the aerial parts used in this study which consisted of leaves and flowers possess more antiplasmodial compounds considering that they manufacture secondary metabolites than the roots used in literature hence the higher antiplasmodial activity observed in this study. This finding that aerial parts of *Pentas lanceolata* showed higher antiplasmodial activity is encouraging as this prevents the plant from over exploitation since aerial parts can be harvested leaving behind the rest of the plant to continue growing, unlike the roots which require uprooting the whole plant.

The roots and aerial parts of *S. hilderbrandtii* had high *in vitro* activity. This agrees with literature values where the roots of *S. hilderbrandtii* have also been reported to have high *in vitro* antiplasmodial activity against D6 clone of *P. falciparum* with an IC_{50} value of 3.8 µg/ml respectively $CHCl_3$ extract (Koch *et al*., 2005). This confirms the potential of this plant as a future antimalarial.

The methanol extracts of roots of *S. hilderbrandtii* had significantly (P=0.036) higher antiplasmodial activity than the aerial parts. This trend was similar for water extracts, where the roots had higher antiplasmodial activity than the aerial parts. This could be due to the fact that roots act as storage organs in most plants (Babitha *et al.,* 2002) and hence

store phytochemical compounds in high concentration manufactured in the leaves and transported to the roots for storage. The knowledge that roots of *S. hilderbrandtii* have higher antiplasmodial activity than the aerial parts is encouraging since the roots were also found to be higher yielding than the aerial parts hence more available for use.

Methanol and water extracts of *Ximenia americana* had good *in vitro* antiplasmodial activity against D6 strain of *Plasmodium falciparum.* The methanol extract of *X. americana* had higher activity than the water extract and higher activity than Chloroquine (control) against D6 strain. And moderate antiplasmodial activity against Chloroquine resistant W2 strain of *P. falciparum*. However it could not compare with artemisinine (control) (IC_{50}, 0.962µg/ml). This is because artemisinine is a pure compound that has been isolated from *Artemisia annua.* Furthermore the high activity demonstrated for *X. americana* water extract against D6 strain of P. *falciparum* confirms its use in traditional medicine in the treatment of malaria where water is mainly the solvent used in formulation of the drugs. This has been reported by Diallo *et al.,* (2002). Therefore the results in this study confirm the high potential of this plant as a future antimalarial. And partially validates its use as an antimalarial as used in the traditional setting.

Generally the plants tested were not as active as the reference drug artemisinine (IC_{50} 0.962 µg/ml for D6 strain and 3.56 µg/ml for W2 strain of *P. falciparum*). This could be due to the fact that the extracts were heterogeneous mixture of different compounds. Isolating pure compounds from the extracts may give similar IC_{50} values as the pure compounds like Artemisinine. Most of the plant extracts which have IC_{50} values often 5-10 µg/ml are potential source of antimalarial compounds (Nkunya *et al.,* 1991). *Artemisia*

annua for example has IC_{50} values of 3.9µg/ml (Philipson and Wright, 1991) and has been a major source of antimalarial compounds. Most of the extracts in this study demonstrated IC_{50} values within this range and therefore have high potential as future antimalarials.

In the *in vivo* test, it would be considered that death of infected treated animals occurring earlier than those infected and untreated would probably be due to toxicity of the extract. The fact that all the extracts prolonged the survival time of infected mice when compared with infected untreated mice showed that there was some intrinsic antimalarial activity in the extracts. There was significant parasite density reduction ($p=0.012$) in animals treated with most of the plant extracts compared to the ones treated with the negative control, 10% tween 80.

The reduced peak parasitaemia on day 4 in all treated groups compared to the negative control group was an indicator of antimalarial potential of the extracts. The *in vivo* model was employed for this study because it takes into account the possible prodrug effect and possible involvement of the immune system in eradication of infection (Waako *et al*., 2005).

In this study seven of the ten extracts subjected to *in vivo* antmalarial test at 500mg/kg body weight showed significant Chemosuppression ($p=0.032$) on day 4 ranging from 30.29% to 64.92%, which partially validates their use in ethnomedicine for treatment of malaria. Notably that all the water extracts were active against *P. berghei* in mice, while

for methanol extracts only aerial parts of *Pentas lanceolata* and stem bark of *Ximenia americana* were active with Chemosupression of 64.92% and 50.81% respectively. This explains why these plants are used in traditional therapy where water is mostly the solvent used in formulation (Gessler *et al.*, 1995).

The aerial parts of *F. africana* methanol extract were inactive in the mouse model with chemosuppresion of 27.88%± 3.47, though it had a high *in vitro* antiplasmodial activity (IC_{50} 1.841 µg/ml). This could be due to biotransformation of the constituents or poor bioavailability and transport of the active compounds *in vivo* (Muregi *et al.*, 2007). Water extract of *F. africana* (aerial part) exhibited mild antimalarial activity with Chemosuppression of 30.80%±2.80 during *in vivo* test, despite lower *in vitro* antiplasmodial activity with and (IC_{50} 17.035 µg/ml) against D6 strain of *P. falciparum*. It is possible that the compounds were converted to active metabolites in mice (Muregi, 2007). This finding differs with literature where earlier investigation by Muthaura, (2007), revealed *F. africana* to have a Chemosuppression of 61.85±4.61% and 43.16±1.69% for methanol and water extracts respectively.

The presence and quantities of bioactive compounds in plants are influenced by several factors including seasons of the year, georeference, plant-part used, intra-species variations and plant age (Weenen *et al*., 1990), and this may explain the discrepancies observed in this study and literature. Also considering that only aerial parts were used in this study as opposed to whole plant parts as used to obtain results in literature is another cause of the difference. Moreover the specimens were collected from different

geographic locations, at different seasons and possibly different ages since the plants were collected from the wild.

Aerial parts of *Pentas lanceolata* also showed good activity *in vivo* with chemosuppression of 64.92±0.14% for methanol extract and 41.04 ± 2.02% for water extract at 500mg/kg body weight. This consistency in *in vitro* antiplasmodial activity and *in vivo* antimalarial activity together with the observed lack of cytotoxicy in Vero E99 cells and the lack of toxicity in mice shows and confirms that *P. lanceolata* is an effective and safe folklore antimalarial herb. This therefore validates its use as antimalarial as already used by traditional health practitioners in the rural set up. The knowledge that the methanol extract is more active *in vivo* than water extract is useful since traditional health practitioners mostly use water in preparation of herbal concortions (Muthaura *et al*., 2007).

The roots and aerial parts of *S. hilderbrandtii* methanol extracts were inactive *in vivo* with chemosupression of 12.63±1.26%, and 17.53±2.07% respectively, despite the high *in vitro* antiplasmodial activity (IC_{50} of 2.12µg/ml, and 3.153µg/ml) respectively. Drugs active *in vitro* may not necessarily be active *in vivo* (Gassler *et al.*, 1995). Lack of activity of extracts *in vivo* which were active *in vitro*, might be due to low absorption or because structures necessary for activity may be altered by metabolic processes in mice (Muthaura, 2007).

However the lack of activity *in vivo* does not imply that *S. hilderbrandtii* is disgualified as an antiamalarial since both aerial parts and roots of *S. hilderbrandtii* was able to show good *in vitro* antiplasmodial activity even against Chloroquine resistant W2 strain of *P. falciparum* (IC_{50}, 12.688± 4.69 µg/ml and 14.851±3.37 µg/ml respectively). Furhermore herbal medicines may act by more than one mechanism like having an indirect effect on the immune system as described by Rasoanaivo *et al.,* (1992).

Interestingly water extracts of the roots and aerial parts of *S. hilderbrandtii* were active *in vivo* with chemosupression of 30.29% and 33.89% respectively though they had lower *in vitro* antiplasmodial activities against D6 strain of *P. falciparum* than the methanol extracts (IC_{50} 4.096 µg/ml, root and 18.004 µg/ml, aerial parts). This could be due to the fact that the compounds acted as prodrugs which were converted to active metabolites by metabolic processes in mice (Muregi *et al.,* 2007). This is a possible explanation as to why most medicinal plants are traditionally taken with water as the solvent of extraction (Gachathi, 1989; Kokwaro, 1993). This is also the reason why *S. hilderbrandtii* is consistently used as an antimalarial in traditional medicine.

Methanol and water extracts of *Ximenia americana* had good *in vivo* antimalarial activity with chemosupression of 50.81% and 54.87% respectively. This same plant had high *in vitro* antiplasmodial activity against D6 strain of *Plasmodium falciparum.* It is worth noting that the water extract of *X. americana* has higher activity in mice than the methanol extract yet the reverse was true *in vitro*. This is probably due to biotransformation of chemical constituents into more active metabolites in mice (Muregi,

et al., 2007). This knowledge is encouraging since water is mainly the formulation used in the traditional preparation of herbal remedies. These findings are supported by reports of antiparasitic activity of *Ximenia americana* by Maikai, (2010). Furthermore its traditional use as an antimalarial has been reported by Gronhaug *et al.,* (2008) and Diallo *et al.,* (2002). Therefore the results in this study confirm the high potential of this plant as a future antimalarial. And partially validates its use as an antimalarial as used in the traditional setting.

In most cases, remarkable suppression of parasitassemia by extracts translated into a longer mouse survival. For example stem bark of *Ximenia americana* and aerial parts of *Pentas lanceolata* methanol extracts were associated with the highest mean survival time at 11.60 and 11.40 days respectively, compared to infected untreated mice which had a mean survival time of 8.60 days and had the highest chemosuppression. Supprisingly methanol extract of *F. africana* was inactive with a chemosuppression of 27.88% but provided a mean survival time of 10.6 days. This implies that other than direct parasiticidal effects, this plant extract may possess other pharmacological benefits to the hosts, such as acting as analgesics, antipyretics or as immune stimulators (Dahanukar *et al.,* 2000).

On the contrary water extract of *X. americana* had a good activity with a Chemosuppression of 54.87% but had a shorter mean survival time of 9.3 days. This indicates that the extracts act on the blood stages of the parasites, suggesting that the bioactive compounds in these plant extract may have a short half life. Some antimalarial

drugs including artemisinine based derivatives are known to be fast acting, and to have a short half life (Muregi *et al*., 2007).

In acute toxicity assay no deaths were observed for eight of the ten extracts at the highest concentration tested which was 5000 mg/kg body weight, indicating that the plant extracts were not toxic at that concentration. Another pointer to the safety of most of the samples tested is that all the animals that received the extracts stayed alive for the entire period of the 4 day suppressive test. According to Jutamaad *et al*., (1998) if the test mice die before day 5 the cause of death is usually attributed to the effect of the test drugs rather than the parasites.

Moreover the doses of the water and methanol extracts orally administered up to 5000mg/kg body weight were much higher than those taken as infusion by people. Mortality was recorded for *X. americana* water extract. The same extract was tolerated and had good antimalarial activity at a dose level of 500mg/kg with Chemosuppression of 54.87%. It was also not cytotoxic against Vero E99 cell lines with a CC_{50} value of ≥100µg/ml. It can therefore still be used as an antimalarial administered at lower safe doses.

The methanol extract of *Fuerstia africana* had good antimalarial activity with no signs of acute toxicity in swiss albino mice. However it exhibited moderate cytotoxicity with CC_{50} of 63.45µg/ml on Vero E99 cell lines *in vitro*, though it had a high selectivity index of 34.465, this indicates selective toxicity to malaria parasite. The selectivity index (SI) is

the ratio of cytotoxicity to biological activity. It is generally considered that biological efficacy is not due to the *in vitro* cytotoxicity when SI ≥ 10 (Vonthron- Senecheau *et al.*, 2003). This means that the effectiveness of this plant as an antimalarial is not due to cytotoxicity.

In vitro cytotoxicity does not necessarily mean an extract cannot be used in human ethnomedicine since safe non toxic compounds can still be isolated (Kokwaro 1993; Baraza *et al.,* 2008). This demonstrates the potential of this plant as an antimalarial administered at safe doses. The rest of the extracts were not cytotoxic on Vero E 99 cell lines with CC_{50} values ≥100µg/ml, suggestive of the safety of the medicinal plants that have been used over many years.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In this study a demonstration of antiplasmodial potential and safety of the four selected medicinal plants used in Kenya was achieved. This study supports the use of *P. lanceolata* and *Ximenia americana* as traditional antimalarials.

The lack of cytotoxicity of most plants extracts suggests the safety of the medicinal plants that have been used for a long time in the treatment of malaria. Although aerial parts of *F. africana*, methanol extract had moderate cytotoxicity it can still be used in lower safer doses. The fact that most of the plant extracts were not toxic to mice at 5000mg (5g) body weight validates their use in high doses as used in traditional medicine. Since *X. americana* water extract showed mortality of less than 50 % of the population, it can still be used as an antimalarial at safe lower doses in Traditional medicine.

6.2 Recommendations

I. Medicinal plants with high antiplasmodial activity like and *X. americana* and *Pentas lanceolata* should be pursued further for evaluation, large scale production and formulation of the drugs.

II. Plants with good antimalarial efficacy like *P. lanceolata, X. americana* should be validated for use in areas where access to conventional medicine is limited since their safety is guaranteed.

III. Plants with high antimalarial potential should be domesticated to avoid over exploitation and possible extinction of plant species with medicinal value.

IV. Bio active compounds in aerial parts of *Fuerstia africana* methanol extract should be isolated and their structure used in formulation of new drugs.

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APPENDICES

Appendix I: Calculation of % parasitaemia, and growth rate

Percentage parasitaemia % P = No. of parasitisized erythrocytes

(RBC per field) × (fields counted)

Growth rate (GR) is calculated at every 48 hour cycle using the formula:

$GR = (P_f/P_i \times D)\ 2^{/d}$

Where,

P_f - is the final parasitaemia

P_i-initial parasitaemia before dilution

D- Is the dilution factor

d- Is the number of days since culture was diluted

Appendix II: Test drug (plant extract) and standard drug calculation for *in vitro* assay

Methanol or water extract weighing 0.045 was dissolved to a final volume of 4ml (stock solution of 2500 µg/ml). This was calculated using the formula: $C_1V_1=C_2V_2$

Where C_1= Initial concentration

V_1= Initial volume

C_2= Final concentration

V_2= Final Volume

Taking into account that the volume of each drug in each well was 25 µl (initial volume), the final volume in each well was 250 µl, this stock solution was meant to give the first row concentration of 250 µg/ml.

Therefore;

$$C_1 \times 25\ \mu l = 250\ \mu g/ml \times 250\ \mu l$$

$$= 2500\ \mu g/ml$$

Similarly Chloroquine diphoshate was prepared so that the plates first row concentration was 0.1 µg/ml (1 µg/ml stock solution, C_1), sterilized and stored at -20^0C.

$C_1 \times 25\ \mu l = 0.1\ \mu g/ml \times 250\ \mu l$

$C_1 = 0.1\ \mu g/ml \times 250\ \mu l/25\mu l$

$C_1 = 1\ \mu g/ml$

Appendix III: Calculation of CMS, test culture and 1.5 % haemotocrit needed for setting of plates

Taking the % parasitaemia of the test cultures (V_1) to have been 4 % and the number of plates to be set to have been 1 (n=1), the following calculations were done (recall that the cultures were maintained at 5ml and 6 % hematocrit)

$$C_iV_i=C_fV_f$$

Where: C_i and C_f = initial and final concentrations, respectively

$V_{i\ and}$ V_f = initial and final volumes, respectively

C_i = 4%

C_f = 0.4%

The volume of plate ($V_{f)}$ was calculated as follows: (96 wells is approximately 100 wells

V_f = 1 plate ×100 wells × 200µl (volumes of culture per well)

= 20000µl

= 20ml

The volume of test culture (5ml, 6% hematocrit) which was used (V_1) was calculated as follows:

$C_iV_{i\ =}$ C_fV_f

4% ×V_1 =0.4% × 20ml

V_1 =0.4 × 20ml/ 4 % = 20ml

Since 5ml has 5% hematocrit or 6/100 ×5ml = 0.3ml (100% RBC)

2ml culture has 0.12 (100% RBC)

To adjust hct to 1`.5% of V_f 1.5/100 ×20µl = 0.3ml (100% RBC)

But the V_1 (2ml) has 0.12 ml (100% RBC) and 0.3ml -0.12ml= 0.18ml (100% RBC) were required. This required the addition of 50% RBC. Since the remaining 0.18ml hct was 100% RBC, 0.18×2 = 0.36ml of 50% RBC was needed.

The final volume of 20ml needed was achieved by addition of CMS to 20ml test culture and 0.36ml of (50% RBC)

CMS needed =20mls – (2=0.36ml)

= 20mls -2.36 ml

= 17.64ml

This means that to set 1 plate using a culture whose % P = 4% you require 17.64ml; CMS, 0.36 ml (50 % RBC) and 2 ml test culture to achieve 0.4% parasitaemia and 1.5 % hct.

For unparasitized red blood cells UPRBC wells H_7-H_2 the CMS and RBC needed was,

For plate (6 wells)

Volume = 6ml × 200µl

= 1200µl

=1.2 ml

1.5% hct =1.5/100 ×1.2

=0.018ml (100% RBC)i

=0.036 (50 % RBC)

CMS = 1.2 – 0.036

= 1.164ml

0.036ml of 50% RBC were mixed with 1.164ml CMS and 200µl were aliquoted in to well H_7 to H_2 using a multi-channel pipette. The same procedure and calculation were done for n number of plates n =2, 3…. etc.

Appendix IV: Calculation of % parasitaemia, and % Chemosuppression

$$\text{Percentage parasitaemia (\% P)} = \frac{\text{No. of parasitized erythrocytes}}{\text{(RBC per field) x (fields counted)}} \times 100$$

Percentage Chemosuppression is calculated using the formula:

$$\text{\% Chemosuppression} = \frac{(A - B)}{A} \times 100$$

Where,

A- Is the parasite density of the Negative control

B- Is the parasite density of test extracts and positive control.

Appendix V: Preparation of buffers and solutions

Phosphate saline glucose buffer (PSG)

5.392g di-sodium hydrogen phosphates (Na_2HPO_4)

0.312g sodium di-hydrogen phosphate ($NaH_2PO_4.2H_2O$)

1.7g Sodium chloride (NaCl)

10.0g D-glucose

The mixture is dissolved in 1 litre of distilled water, sterilized by autoclaving at 121^{o} C for 15 minutes and stored in the dark at 4^{o} C.

Giemsa stock Solution.

3g Giemsa powder

300ml methanol

200ml 100% glycerol

The solution is mixed thoroughly for 45-60 minutes and stored at room temperature.

70% tween 80 and 30% ethanol (stock solution)

7 parts of tween-80 is mixed with 3 parts of analytical ethanol.

10% tween 80 (working solution).

The stock is mixed with distilled water at a ratio of 1:9. 1 part of stock solution is mixed with 9 parts of water.

Appendix VI: Schematic presentation of a test plate done in duplicate

	1		**2**		**3**		**4**		**5**		**6**	
100%												
50%												
25%												
12.5%												
6.25%												

3.125%												
1.525%												
8 wells of control (**PRBC**) without drug									**UPRBC**			

KEY

PRBC: Parasitized red blood cells

UPRBC: Unparasitized red blood cells

1-6: Six drugs set in duplicate at different concentrations

Appendix VII: IC_{50} values of methanol extracts and the control Artemisinine for D6 strain of *P. falciparum*

Extract	df	P values
S. hilderbrandtii (aerial parts)	2	0.005
S. hilderbrandtii (roots)	2	0.017
F. africana (aerial parts)	2	0.010
P. lanceolata (aerial parts)	2	0.094
X. americana (stem bark)	2	0.040

Appendix VIII: IC_{50} values of methanol extracts and the control Chloroquine for D6 strain of *P. falciparum*

Extract	df	P values
S. hilderbrandtii (aerial parts)	2	0.053
S. hilderbrandtii (roots)	2	0.013
F. africana (aerial parts)	2	0.090
P. lanceolata (aerial parts)	2	0.023
X. americana (stem bark)	2	0.062

Appendix IX: IC_{50} values of water extracts and the control Artemisinine for D6 strain of *P. falciparum*

Extract	df	P values
S. hilderbrandtii (aerial parts)	2	0.011
S. hilderbrandtii (roots)	2	0.055
F. africana (aerial parts)	2	0.020
P. lanceolata (aerial parts)	2	0.017
X. americana (stem bark)	2	0.019

Appendix X: Differences in IC_{50} values of water extracts and the control Chloroquine for D6 strain of *P. falciparum*

Extract	df	P values
S. hilderbrandtii (aerial parts)	2	0.009
S. hilderbrandtii (roots)	2	0.001
F. africana (aerial parts)	2	0.023
P. lanceolata (aerial parts)	2	0.009
X. americana (stem bark)	2	0.033

Appendix XI: IC_{50} values of water extracts and the control Artemisinine for W2 strain of *P. falciparum*

Extract	df	P values
S. hilderbrandtii (aerial parts)	2	0.000
S. hilderbrandtii (roots)	2	0.003
F. africana (aerial parts)	2	0.002
P. lanceolata (aerial parts)	2	0.008
X. americana (stem bark)	2	0.002

Appendix XII: IC_{50} values of water extracts and the control Chloroquine for W2 strain of *P. falciparum*

Extract	df	P values
S. hilderbrandtii (aerial parts)	2	0.000
S. hilderbrandtii (roots)	2	0.552
F. africana (aerial parts)	2	0.005
P. lanceolata (aerial parts)	2	0.067
X. americana (stem bark)	2	0.022

Appendix XIII: IC_{50} values of methanol extracts and the control Artemisinine for W2 strain of *P. falciparum*

Extract	df	P values
S. hilderbrandtii (aerial parts)	2	0.078
S. hilderbrandtii (roots)	2	0.028
F. africana (aerial parts)	2	0.004
P. lanceolata (aerial parts)	2	0.012
X. americana (stem bark)	2	0.005

Appendix XIV: IC_{50} values of methanol extracts and the control Chloroquine for W2 strain of *P. falciparum*

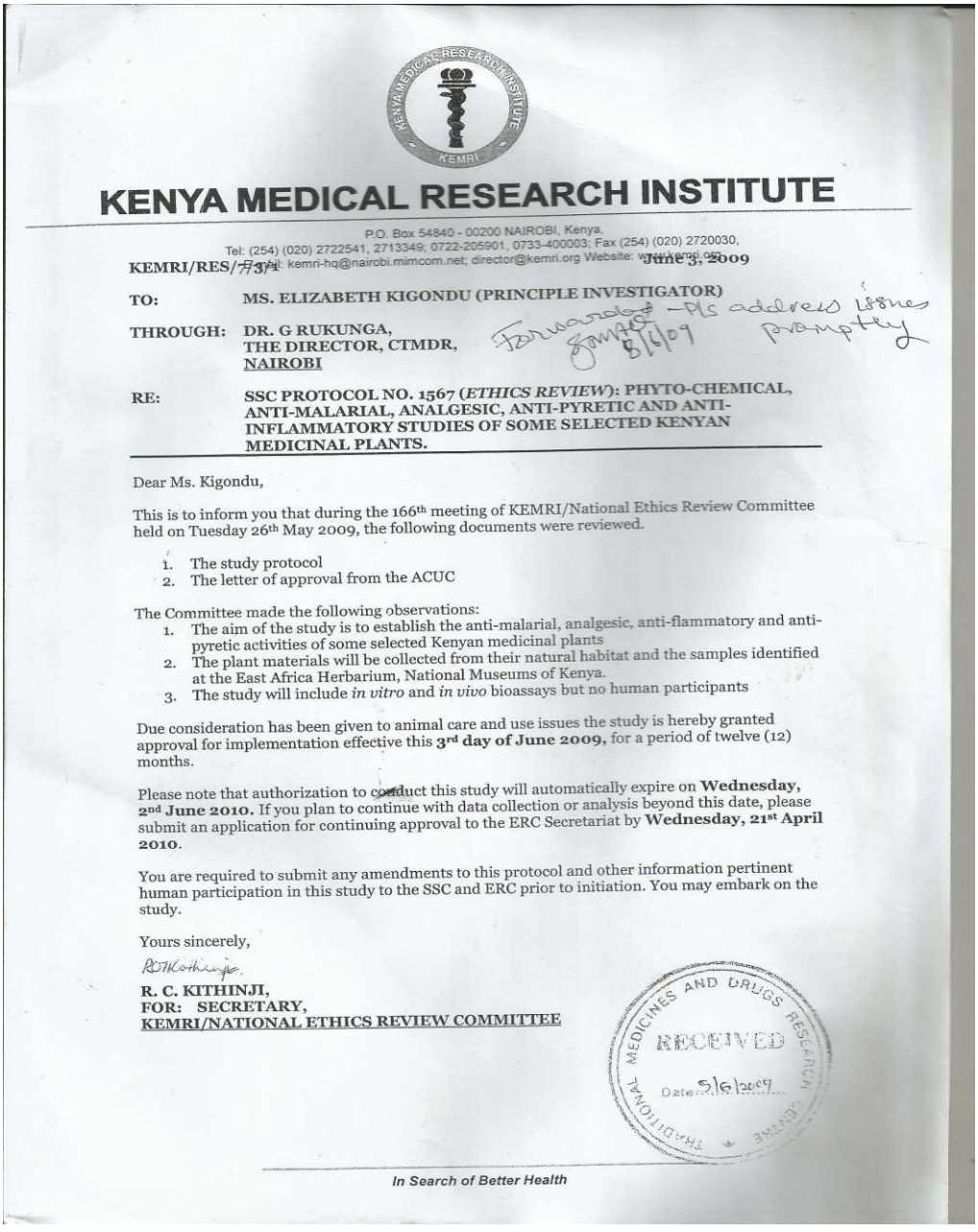
Extract	Df	P values
S. hilderbrandtii (aerial parts)	2	0.004
S. hilderbrandtii (roots)	2	0.002
F. africana (aerial parts)	2	0.000
P. lanceolata (aerial parts)	2	0.001
X. americana (stem bark)	2	0.017

Appendix XV: Culture medium RPMI 1640 and 96 well cell harvester used for *in vitro* antiplasmodial assay

Plate I: Culture medium RPMI 1640 in a sterile laminar floor hood in malaria culture laboratory, Center for Biotechnology Research and Development, KEMRI, Nairobi,Kenya. (Source : Author, 2013)

Plate II: 96 well cell harvester in Phytochemistry laboratory, Center for Traditional Medicine and Drug Research, KEMRI, Nairobi, Kenya. (Source: Author, 2014).

Appendix XVI: Ethical approval



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KEMRI/RES/7/3/1 June 3, 2009

TO: MS. ELIZABETH KIGONDU (PRINCIPLE INVESTIGATOR)

THROUGH: DR. G RUKUNGA,
THE DIRECTOR, CTMDR,
NAIROBI

Forwarded – Pls address issues promptly
8/6/09

RE: SSC PROTOCOL NO. 1567 (*ETHICS REVIEW*): PHYTO-CHEMICAL, ANTI-MALARIAL, ANALGESIC, ANTI-PYRETIC AND ANTI-INFLAMMATORY STUDIES OF SOME SELECTED KENYAN MEDICINAL PLANTS.

Dear Ms. Kigondu,

This is to inform you that during the 166th meeting of KEMRI/National Ethics Review Committee held on Tuesday 26th May 2009, the following documents were reviewed.

1. The study protocol
2. The letter of approval from the ACUC

The Committee made the following observations:

1. The aim of the study is to establish the anti-malarial, analgesic, anti-flammatory and anti-pyretic activities of some selected Kenyan medicinal plants
2. The plant materials will be collected from their natural habitat and the samples identified at the East Africa Herbarium, National Museums of Kenya.
3. The study will include *in vitro* and *in vivo* bioassays but no human participants

Due consideration has been given to animal care and use issues the study is hereby granted approval for implementation effective this **3rd day of June 2009,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **Wednesday, 2nd June 2010.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **Wednesday, 21st April 2010**.

You are required to submit any amendments to this protocol and other information pertinent human participation in this study to the SSC and ERC prior to initiation. You may embark on the study.

Yours sincerely,

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

TRADITIONAL MEDICINES AND DRUGS RESEARCH CENTRE
RECEIVED
Date 5/6/2009

In Search of Better Health

Plate III: Ethical approval by the KEMRI's Ethics Review Commitee Nairobi, Kenya. (Source: Author, 2014).