A study of the antimalarial activity of combined plant extracts from medicinal plants used traditionally in Kenya.

By

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U56/88727/2016

A research thesis submitted in partial fulfillment for the award of the degree of Masters of Science in Pharmacognosy and Complementary Medicine, University of Nairobi.

December, 2020

DECLARATION

I, Julia Janet A. Ouma, do hereby declare that this thesis is my original work and that this work has not been presented for the award of any degree to any other university.

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DEDICATION

This work is dedicated to my loving husband, Dr Benard Juma, for your sacrifice, encouragement and guidance, to my children Loch, Kwe and Ng'wono for your perseverance and understanding when I had to be away and to mum, Prof. Josephine Ouma for always reminding me on the need to go back to school.

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ABBREVIATIONS AND ACRONYMS

CC_{50}	Cytotoxic concentration 50%
CMS	Complete medium with serum
CTMDR	Centre for Traditional Medicine & Drugs Research
DMSO	Dimethyl Sulphoxide
HEPES	N-Hydroxyethylpiperazine-N-2-ethanol sulfonic acid buffer
GLP	Good Laboratory Practice
IC_{50}	Inhibition concentration 50%
KEMRI	Kenya Medical Research Institute
KNH/UON	Kenyatta National Hospital/ University of Nairobi
LD50	Lethal dose 50% (Median Lethal Dose)
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate Saline Buffer
RPMI 1640	Roswell Park Memorial Institute (*RPMI*) 1640 Medium
W.H.O	World Health Organization

ABSTRACT

Introduction

Medicinal plants have been in use in the management of disease and maintenance of health by societies for thousands of years. Emerging resistance to presently utilized single molecule drugs has heightened the need to research into plant sources of antimalarial drugs.

Study objectives

The study aimed to explore the antiplasmodial activity of combined plant extracts from six commonly used medicinal plants in Kenya and examine the safety profile of those with the best efficacy.

Study methodology

In vitro tests were carried out against chloroquine resistant *P. falciparum* W2 strain using the [^{3}H] hypoxanthine uptake assay, while *in vivo* antiplasmodial and acute toxicity tests were carried out using *Plasmodium berghei* on mice. Cytotoxicity tests were done using the MTT based colorimetric assay on Vero 199 cells and selectivity index was used to assess safety of the extracts.

Results and discussion

Activity criteria in the *in vitro* assay for unpurified extracts were classified as follows: high at IC_{50} ≤10 µg/ml, moderate at 10–50 µg/ml, low at 50–10 µg/ml and inactive at >100 µg/ml. *Schkuhria pinnata* had the best activity of IC_{50} 10.81 µg/ml *in vitro* of all the extracts tested. Extracts from *Flueggea virosa, Boscia angistifolia and Zanthoxylum chalybeum* had moderate activity of 19.03, 20.26 and 37.69 µg/ml respectively. *Toddalia asiatica* extract had low activity having IC_{50} above 50 µg/ml whereas *Carissa edulis* was found to be inactive having an IC_{50} above 100 µg/ml. The combinations of extracts involving *Z. chalybeum* showed the most activity with IC_{50}'s below 10 µg/ml. Antagonism was observed with combinations such as *F. virosa* with *S. pinnata*, *S. pinnata* with *B. angustifolia*, *B. angustifolia* with *T. asiatica*, *C. edulis* with *B. angustifolia* and lastly, *C. edulis* with *T. asiatica*.

The combination of *S. pinnat*a and *T. asiatica* extracts exhibited the best activity on *in vivo* tests having a parasite growth suppression of 81.3% at a dose of 500 mg/kg, which was comparable to 87.2% of Artemether at a dose of 10 mg/kg body weight as the positive control. Singly, the two plant extracts showed good activity 65.2 and 58.9% at doses of 250 mg/kg and 500 mg/kg respectively. On cytotoxicity testing, *S. pinnata* and *T.asiatica* had CC_{50} of 102.93±5.96 µg/ml and>1000 µg/ml respectively and selectivity indices of 9.52 and 15.24 respectively indicating good therapeutic potential due to their safety margins.

Conclusion and recommendation

The results obtained from both *in vitro* and *in vivo* tests support the use of the plants selected in traditional treatment of malaria. A combination of methanol fruit extract of *T. asiatica* and methanol whole plant extract of *S. pinnata* showed promising parasite growth suppression against *P. berghei* that was comparable with Artemether and was synergistic on *in vitro* testing against chloroquine- resistant W2 strain of *P. falciparum*.

The combination of *S. pinnata* and *T.asiatica* extracts can be considered for formulation into a polyherbal antimalarial drug but would require further investigation such as effective dosage, chronic toxicity and phytochemical screening to determine the compounds responsible for activity.

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INFORMATION

The use of medicinal plants in treatment of diverse diseases and for maintenance of health by different traditional societies is well known. Most of the plants were traditionally used in their crude form as decoctions which were, drunk, inhaled, bathed with or used as poultices. Knowledge on these plants was passed down through oral narration. In modern times, active compounds have been isolated from plant extracts leading to discovery of drugs in allopathic medicine such as quinine, morphine, artemesinin, atropine, pilocarpine, cocaine and many others, some of which are still in use to date (Balunas and Kinghorn, 2005). WHO avers that up to 85% of people living in third world countries use alternative medicine as remedies to diverse disease conditions (WHO, 2011).

One of the conditions that has benefited greatly from research on medicinal plants is malaria, which informs the current study.

1.1.1 Statistics

Malaria being a tropical disease is of great public health concern. It has high morbidity and mortality, and affects about 3 billion of the world population, in particular those residing in the tropics such as sub-Saharan Africa, Asia, and Latin America. A disproportionate share of 90% of the global malaria burden is carried by Sub-Saharan Africa which also accounts for 92% of malaria deaths (WHO, 2016). In 2015, 303,000 malaria deaths occurred in children under the age of 5, which makes up to 70% of the global total. It is reported that every two minutes worldwide, a child dies of malaria; hence the disease requires renewed effort and attention in its prevention and treatment (WHO, 2016).

Due to the high prevalence, malaria impacts African nations negatively. It is estimated that nations lose income in the tune of 12 million USD a year. This is evaluated in terms of

healthcare costs, absenteeism from work and school, decreased productivity due to cerebral malaria brain damage, loss of investments and tourism income (Gallup and Sachs, 2001)

1.1.2 Life cycle of Malaria parasite

The female *Anopheles* mosquitoes are the vectors for the Plasmodium parasite responsible for malaria. They inject the malaria parasite as they feed on human blood. The parasites enter circulation directly or through the lymphatic system after which they invade the hepatic cells, where they differentiate and multiply and thereafter, are released into the bloodstream as merozoites. These, then infect red blood cells and further multiply leading to lysis of the cells. This process releases more merozoites to infect other red blood cells. The erythrocytic stage of the parasite is responsible for the clinical manifestations of the disease. Some of the merozoites differentiate into gametocytes which when released into the blood stream, are taken up by the feeding mosquito to complete the human stage cycle. Once in the mosquito, sexual reproduction occurs where the male gametocytes penetrate the female gametocytes resulting in zygote formation. These in turn become motile and elongate, penetrate the gut wall and develop into oocytes. The oocytes grow and rapture, releasing sporozoites which travel to the mosquito's salivary glands, awaiting inoculation into the next human host. This completes the malaria cycle.

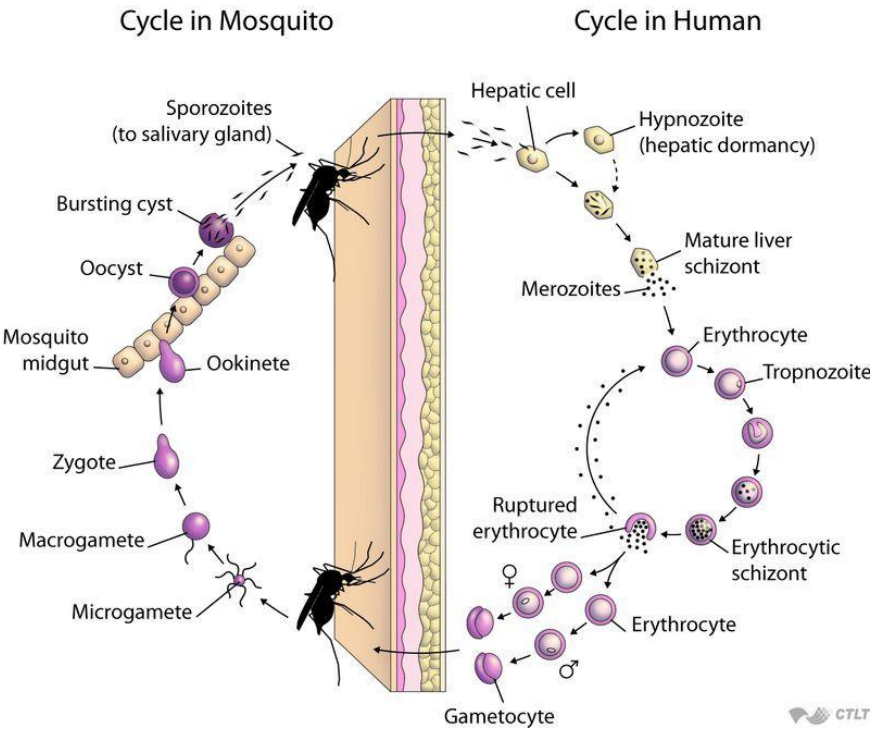

Figure 1: Lifecycle of the *Plasmodium* showing the development stages in human and mosquito

(Image from https://www.malariasite.com/life-cycle)

The four parasite species responsible for the human disease are: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, with *P. falciparum* and *P. vivax* being responsible for most severe malaria infections. Clinical symptoms of malaria range from fever, tiredness, vomiting, and headaches to severe symptoms such as jaundice, seizures, coma, or death.

1.1.3 Conventional treatment and resistance challenge

The management of malaria involves different approaches, both prophylactic and curative. Examples of drugs that have been used in treatment include: quinine, chloroquine, mefloquine, primaquine, pyrimethamine, artemisinin derivatives, lumefantrine, halofantrine, doxycycline and sulfadoxine.

Quinine was the first drug isolated from Cinchona bark for modern treatment of malaria (Willcox, Merlin 2004). However, the bark extracts had been in use in South America for many centuries prior for management of fevers and other conditions. Due to several adverse reactions to quinine, the WHO removed it as a first line treatment of malaria and is reserved for severe malaria in the absence of artesunate (a synthetic derivative of artemesinin) (W.H.O, 2006) During the period of World War II, chloroquine, a synthetic derivative of quinine, found tremendous use as a first line antimalarial drug due to its relative safety and cost effectiveness. Its widespread use through many years led to development of resistance in *P. falciparum* and *P.vivax,* which was first noted in Southeast Asia, Oceania, and South America in the late 1950s and early 1960s and eventually spread to nearly all tropics and subtropics where *P. falciparum* malaria is transmitted (Payne, 1987). The parasites have also developed resistance to several other drugs such as: quinine, the antifolates, pyrimethamine and suphadoxine, atovaquone, mefloquine and halofantrine (Cui *et al.*, 2015).

Current treatment recommended by WHO involves the use of combination therapy with artemesinin derivatives as the base. Artemesinin is highly efficacious in treatment of acute malaria but is short acting. Its use as monotherapy is highly discouraged and hence it is used in combination with longer acting agents such as lumefantrine to allow shorter duration of treatment and reduce resistance selection to the atemesinin component. According to the WHO status report of December 2019, Pfkelch 13 mutations, which are identified as the molecular markers of partial artemesinin resistance, have been reported as widespread in the greater

Mekong subregion and detected in Guyana, Papua New Guinea and Rwanda (WHO, 2018, Subregion, 2014). A compelling need therefore arises to seek new malaria treatments that would be affordable and convenient to both patients and healthcare providers.

Herbal medicine has been a mainstay of management of malaria and fevers in many indigenous communities for thousands of years without development of resistance. Indeed, artemesinin, which is the current recommended first line treatment of malaria, is derived from a plant *Artemesia annua*, while quinine which is reserved for severe malaria was derived from *Cinchona* plant and had been in use for treatment of fevers by South American cultures for thousands of years. The crude extract of *A. annua* was in use in traditional Chinese society for thousands of years without developing resistance and studies have shown that the use of the whole plant can help overcome parasite resistance (Klayman, 1985).

In Kenya, the use of herbs in management of malaria, either singly or in combination is well documented with studies validating their efficacy against malaria parasite (Gathirwa *et al.*, 2008, Muthaura *et al.*, 2007,Orwa *et al.*, 2013). The use of combinations of different herbs is consistent with the practice of many herbalists and is known to combat drug resistance hence the need to validate this practice and confirm whether combining two or more herbal extracts will result in improved efficacy, antagonism or potentiation against chloroquine resistant strains of *P. falciparum*.

1.2 Problem statement

Malaria remains a serious public health issue with high economic costs due to development of resistance to many of the medicines currently in use. Herbs have been used for generations by different cultures to treat fevers and malaria successfully without development of resistance as exemplified by *Artemesia annua* and Cinchona sp. Herbal practice involves combination of different plant extracts but in most cases, the interaction is unascertained. There is therefore need to research and seek new plant extracts that can be used either singly or in combination as new phytomedicines for the treatment of malaria.

1.3 Study Goals and Objectives

The study aims to investigate possible synergy of crude plants' extracts which had previously demonstrated high activity in literature, against chloroquine resistant Plasmodium parasites, with

the combination demonstrating high activity, being recommended for further preformulation studies.

1.3.1 Specific Objectives

1. To confirm the *in vitro* and *in vivo* efficacy of six plant extracts used traditionally as antimalarial medicine against chloroquine-resistant *Plasmodium* species.
2. To establish the effect of combining plant extracts both *in vitro* against chloroquine resistant *P. falciparum* parasites and *in vivo* against *P. berghei*.
3. To evaluate safety of the combination of extracts with the highest efficacy from the *in vivo* and *in vitro* results.

1.4 Justification of study

Plasmodium falciparum has been known to develop resistance against single entity drugs over time with repeated use, yet such resistance has not been shown with whole herbs. Herbs when used singly have shown significant activity against plasmodium parasite but research has also shown that combining several extract may further increase potency (Gathirwa *et al.*, 2008, Rasoanaivo *et al*., 2011). The research sought to explore the effect of combining two herbal extracts with known antiplasmodial activity *in vitro* against chloroquine resistant *P. falciparum* and *in vivo* against *P. berghei*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Utility of herbal medicine in disease management

Plant parts i.e leaves, roots, stems and fruits have been used for nutrition and health purposes for eons by different cultures. WHO report notes that a large number of the world's populace particularly in third world countries still depends on herbal medicine to meet its healthcare needs and in some settings such as rural areas, these complementary medical practices and practitioners are the main and in some instances, the sole providers of healthcare (WHO, 2013). The demand for traditional medicine has increased the use of herbal medicine even in areas where allopathic treatment is readily available (WHO, 2003). Several reasons are attributed to this increased interest which include, cost effectiveness, cultural acceptability, perception of safety and availability, and is seen as a method of coping with the rise in non-communicable diseases (WHO, 2013). Several African countries for instance Nigeria, Mali, Ghana, South Africa and Zambia, have encouraged the use of herbal medicine in treatment and palliative management of serious diseases including HIV/AIDS and malaria, and are integrating herbal remedies in their healthcare systems (Mahomoodally 2013). In such countries, herbal medicines have been used as the first-line therapy for more than 60% of children with high fever. Research carried out in different parts of Africa and North America, have concluded that up to three quarters of people living with HIV/AIDS use herbal medicine solely or as compliment to allopathic treatment for various ailments (WHO, 2003).

2.2 Malaria- etiology and current management

Malaria is a tropical disease caused by protozoa, Plasmodium sp, which are transmitted by an infected female *Anopheles* mosquito. In 2016, 216 million incidences of malaria and 445,000 deaths were reported in 91 countries worldwide. 90% of the malaria cases and 91% of malaria fatalities occur in Africa with children below the age of five being particularly vulnerable to the infection, illness and death. It is in this age group that more than 70% of all malaria cases are reported (WHO, 2016).

Malaria transmission can be minimized by the use of insecticide- treated mosquito nets and spraying of rooms with residual insecticides, to control the vector. Prophylaxis against malaria is achieved by two complementary methods i.e protection against mosquito bites by sleeping under a mosquito net, wearing clothes that cover most of the body and applying insect repellant on exposed skin and chemoprophylaxis. Prophylactic drugs are recommended for travelers from non-endemic to endemic regions and for expectant mothers living in moderate to high malaria endemic zones.

The current first line therapy recommended for non-complicated malaria is artemesinin based combination therapy (WHO, 2016). Other drugs that have been used in times past include: chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, doxycycline among others. An injectable vaccine has recently been developed to confer partial immunity against malaria parasite in children (Iii and Africa, 2014).

2.2.1 Diagnosis of malaria in traditional African Medicine

Traditional medical practitioners of various communities across Africa observed signs and symptoms presented by their patients without conducting pathological examinations. These included visual, clinical, and biological examinations such as tasting of urine, smelling of sores for decay, divination through throwing of seeds or bones, use of mind-altering herbal drugs, and use of stellar signs and interpretation of dreams (Evans 2009).

Many traditional communities relied on understanding of symptoms to diagnose malaria and distinguish it from other conditions. For example, among the Digo of Kenya, a patient presenting with symptoms such as fever, joint pains, weakness, headache, lethargy, abdominal pain, sneezing, loss of appetite and vomiting pointed to a malaria diagnosis (Nguta *et al.*, 2011). Among the Shangani of Zimbabwe feeling of cold or experiencing goose pimples and headaches were considered as symptoms of malaria (Ngarivhume *et al.*, 2015) while in the Plateau region of Togo, clinical signs included fever, headache, shivering, weakness and lack of appetite (Agbodeka *et al.*, 2016). The accurate identification of malaria by local communities varied from one group to another depending on their level of exposure. In some cases, reference to the utilization of a plant for the treatment of fevers, referred to its use against malaria since fevers are the most obvious symptom of the disease, and symptomatic relief is difficult to separate from

actual cure. Other antimalarial plants used may not have been antipyretic, but offered symptomatic relief against other malarial symptoms such as liver and spleen inflammation, gastrointestinal symptoms, anaemia and 'coated tongue' (Odugbemi et al 2007).

2.3 Herbal medicine as remedy for malaria

Large numbers of plants from different regions have been identified from ethno pharmacological studies as being effective in the treatment of malaria. A literature survey carried out in 2004 established over 1,277 plants from 160 plant families that were used by indigenous communities for management of malaria (Willcox and Bodeker, 2004). This number has increased since then due to increased interest in plants with antimalarial activity. All plant parts such as leaves, barks, roots and fruits of a diverse range of trees and shrubs, have found use in traditional medicine in the prevention and treatment of malaria. One setback observed with herbal medicine however, is that the extracts are very bitter which may hinder compliance with the prescribed dosages. Moreover, most of the plants are harvested from the wild and are facing the danger of extinction due to overharvesting and habitat loss. Traditional healers and herbalists do not generally agree on species of plants and dosages of the concoctions most appropriate for the management of malaria even though some specific plants have been cited as having antimalarial properties from different indigenous communities. (Dharani *et al.*, 2010).

Allopathic medicine has benefited from herbal medicine since quinine and artemesinin which are still in use were isolated from plants and more potent derivatives developed semi synthetically. In the recent past, there were two African plant extracts undergoing Phase II clinical trials, namely *Nauclea pobeguinii* and *Argemone mexicana* (Mexican poppy). *N.pobeguinii* is a plant found in the Congo, and it exhibited parasite clearance and clinical response of 90.3% at day 14 in 65 patients involved in the study (Mesia *et al.*, 2010;2012) *A. mexicana* is found in Mali and it exhibited a cure rate of 89% at day 28 (Graz *et al.*, 2010). Moreover, Africa is endowed with a rich biodiversity that can be explored in developing new phytomedicines. Isolation of phytochemicals and use of single molecule drugs has been the trend in herbal medicine research. Of late however, there is resurgent interest in the medicinal use of crude and standardized extracts within the scientific community (Mahomoodally 2013).

In this research, six medicinal plants that have traditionally been used by diverse communities in Kenya were evaluated for their antiplasmodial efficacy against chloroquine resistant *P. falciparum* and specifically explored for their synergistic effect against the parasite. These plants are further discussed individually.

2.3.1 *Carissa edulis* Vahl

Carissa edulis Vahl belongs to the Apocynaceae family. It is a well-known medicinal plant found in several regions of Kenya. It grows at forest edges, forests, woodlands, on rocky hillsides, clay black cotton soils, dry, moist, low and midlands of altitudes 1,500 to 2,500m. It has been used in traditional treatment for headaches, chest pains, rheumatism, gonorrhea, syphilis and rabies. The parts used include leaves, fruits and root bark. The decoction of the root bark has been used in the treatment of malaria among different Kenyan communities (Kokwaro 1976). The Hausa of northern Nigeria have reported the use of *C. edulis* for the treatment of malaria, sickle cell, epilepsy, and other inflammatory conditions. (Ya'u *et al*., 2008)

Figure 2: *Carissa edulis,* showing ripe fruits and foliage

(image from World agroforestry.org)

A study on the total methanolic crude extract found it to be very active against chloroquine sensitive D6 strain of *P. falciparum,* with an IC_{50} of 1.95μg/ml. A lignin, nortrachelogenin, was isolated from the methanolic root back extract and showed significant antiplasmodial activity (Kebenei, Ndalut and Sabah, 2011).

2.3.2 *Toddalia asiatica* (L) Lam.

Toddalia asiatica belongs to the Rutaceae family and has been used by different communities for the treatment of malaria, fevers, cholera, indigestion, rheumatism, lung disease among other conditions. The root bark is claimed to be more powerful than other parts of the plant which are also used medicinally. Decoctions or infusions of the roots are drunk for the treatment of malaria, fever, cholera, diarrhea and rheumatism (Orwa *et al.*, 2008).

Figure 3:- Image of *Toddalia asiatica* fruit and foliage

(https://commons.wikimedia.org/wiki/File:Toddalia_asiatica_05.JPG)

Previous studies carried out on *T. asiatica* indicated that the ethyl acetate extract of the fruits elicited the highest activity against chloroquine resistant *P. falciparum* strain(W2), followed by the aqueous extract of the root bark (IC_{50} = 1.87 µg/ml, 2.43 µg/ml) respectively. *In vivo* test against *P. berghei*, showed that the ethyl acetate fruit extract (500 mg/kg) and aqueous extract of the root bark (250 mg/kg) reduced parasitemia by 81.34% and 56.8% respectively, with higher doses being less effective (Orwa *et al.*, 2013).

2.3.3 *Flueggea virosa* (Roxb. ex Willd.) Royle

Flueggea virosa is a medicinal plant found in tropical Africa belonging to the Phyllanthaceae family and has been used in treatment of diverse array of disease conditions, singly and or in combination with other herbs. As with most medicinal plants, all parts are used with the root being considered the most active. A root decoction or powder can be taken orally or as a bath to

treat urinary, kidney, liver, bile, venereal diseases, conditions affecting the reproductive system such as testicular inflammation, frigidity, sterility, heavy menstruation and others such as rheumatism and arthritis. The roots and fruits are reported to be effective against snakebite while a root infusion is used to treat malaria. (Tabuti *et al*., 2007)

Figure 4: Image of *Flueggea virosa* from

(http://www.westafricanplants.senckenberg.de/root/index.php)

Different *in vitro* studies have proved the efficacy of *F. virosa* against both chloroquine sensitive and resistant *P. falciparum*. Methanol/water extract of the leaves and roots demonstrated high antiplasmodial activity (IC_{50} = 2 μg/ml, 3 μg/ml) respectively (Kaou *et al.*, 2008). When tested *in vivo* against *P. berghei*, the crude hydro-ethanolic extract (500 mg/kg) and ethyl acetate fraction (500 mg/kg) of the leaves were reported to reduce parasitemia by 77.38% and 86.09% respectively. (Singh *et al.*, 2017).

2.3.4 *Schkuhria pinnata* (Lam.) Thell.

Schkuhria pinnata is pioneer annual herb that belongs to the Asteraceae family and occurs extensively in the tropics. It is found on arable and disturbed ground such as roadsides and ploughed lands. The use of *S. pinnata* by different indigenous communities is well documented. For example, among the Langi of Northern Uganda, it was used as a single herb or in combination with other herbs such as Aloe spp and *Baccharoides adoensis* where the leaves would be boiled and filtered and the decoction drunk three times a day for the treatment of malaria. (Anywar *et al.*,2016). The traditional use of *S. pinnata* for the management of malaria

has been validated by several *in vitro* and *in vivo* tests. In a study by Muthaura *et al*, 2007, *S. pinnata* demonstrated high antiplasmodial activity (IC_{50} < 5 µg/ml) against both chloroquine sensitive (D6) and resistant (W2) *P. falciparum* strains.

Figure 5: Image of *Schkuhria pinnata*

(http://publish.plantnet-project.org)

2.3.5 *Zanthoxylum chalybeum* Engl.

Zanthoxylum chalybeum (Rutaceae) is extensively used in herbal medical practice among different communities in Kenya and goes by different names such as Mukenea or Mukanu (Kamba), Roko (Luo), Oloisuki (Maasai), Loisugi or Loisuki (Samburu), Mjafari (Swahili) among others. The stem and root bark decoctions have been used in the treatment of malaria, fevers, headache, sickle cell disease, respiratory tract ailments and intestinal problems among others (Kokwaro 2008). Methanolic root bark extract showed high antiplasmodial activity, having an IC_{50} of 3.65 µg/ml (Rukunga *et al.*, 2009). It has found use traditionally in sickle cell anaemia where the decoction is administered for life. The root bark extract has a benzoic acid derivative which has membrane stabilizing effects and may be responsible for the anti-sickling effects. In a repeat-dose effect study, the extract was shown not to negatively affect haemoglobin formation or increase erythrocyte breakdown even at high doses that would lead to anaemia for most drugs (Ogwang *et al*., 2008)

Figure 6: The spiny stem of *Z. chalybeum* (http://tropical.theferns.info)

Figure 7: Flowers of *Zanthoxylum chalybeum* (http://tropical.theferns.info)

2.3.6 *Boscia angustifolia* A.Rich.

Boscia angustifolia of the Capparidaceae family is found in deciduous woodland and wooded grassland, up to an altitude of 2000m, in drier regions with 200–800 mm annual rainfall. It is often found on stony or rocky soils, but also on laterite and loamy soils, sometimes in dry river beds. It is common on termite mounds. (Orwa et al,.2009) The bark decoctions or infusions are taken to treat malaria, hyperthermia, mumps, dysentery, venereal diseases and psychiatric disorders, and as emetic and anthelmintic, whereas non-specified bark preparations are applied externally against fever, hyperthermia and swollen feet, and for treating wounds. The smoke of burning bark is used in the treatment of neuralgia, inflammations and ophthalmia. Root infusions

are applied as an enema to treat constipation in children, and root decoctions are taken by women to promote fertility (Burkill, H.M., 1985). *In vitro* antiplasmodial studies carried out by Muthaura and coworkers found the water extract of *B. angustifolia* very active against the chloroquine sensitive and chloroquine resistant strains of *P. faciparum* with an IC_{50} of 1.42 μg/ml and 4.77 μg/ml respectively (Muthaura 2007b)

Figure 8: Image of immature fruits of *Boscia angustifolia* (from https://www.feedipedia.org)

2.4 Use of combination of extracts to enhance efficacy

The principle of using combination therapy is based on there being a low chance of developing resistance at the same time to two chemotherapeutic agents having independent modes of action and is in the order of once in 10^{12} treatments (Bloland, Ettling and Meek, 2000)**.** Current conventional antimalarial therapy therefore, prefers combinations for example; Malarone® (atovaquone- proguanil) and artemether-lumefantrine.

Using combination of herbs is an elementary principle of western herbal medicine, traditional Chinese medicine (TCM), African traditional medicine and Ayurveda. When plants extracts are combined, the outcomes can be complicated and unpredictable since different complex interactions can take place. The most beneficial interaction would be synergy or potentiation. However, due to the presence of multiple chemical entities there can also be antagonism and increased toxicity. Synergy is a "phenomenon where two or more agents act together to produce an effect greater than would have been produced from a consideration of individual contribution"

whereas potentiation refers to a phenomenon where an inactive agent causes the active agent to elicit a greater effect than on its own. (Evans, 2009). One advantage of using combination of extracts is that lower dose of the herbal preparation would be needed to achieve the desired pharmacological effect, thus reducing the risk of adverse events.

Previous studies on combinations of some Kenyan plants with antiplasmodial activity have shown synergy, both *in vitro* and *in vivo* (Gathirwa *et al.*, 2008, Rukunga *et al.*, 2009). In these studies, the antiplasmodial activity of combinations of different plants indicated high parasite suppression of up to 95% when administered parentrally. It was also noted that mice treated with single extracts survived for a shorter time than those treated with chloroquine while those treated with a combination of two extracts could match the survival time of the positive control group. Certain combinations such as *L. schweinfurthii* and *B. salicifolia* and *B. salicifolia* and *S. birrea* outlived chloroquine treated mice (18.2, 18.0 and 17.9 days survival period, respectively) (Gathirwa *et al.*, 2008). A study carried out on a widely prepared and utilised antimalarial decoction in Nigeria consisting of *Mangifera indica, Alstonia boonei, Morinda lucida* and *Azadirachta indica* (MAMA), indicated synergy of the different components at different ratios (Odediran *et al*., 2014). A study by Kebenei and others, demonstrated high synergism of a combination of *Carissa edulis* and A*rtemisia annua* total extract in different ratios (Kebenei, Ndalut and Sabah, 2011). The current study selected different sets of plants that had not been combined previously.

Moreover, the use of more than one herbal extract is in line with traditional practice of herbalists where they combine different plants in the management of malaria and other condition (Mills and Bone, 2000). A preclusive step in evaluation of synergistic interaction is to test the effect of individual extracts alone and also in combination. This would give a pointer to synergy or antagonism though it would not be known which compounds are responsible for the interaction. (Evans, 2009).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Plant selection

An existing database at the Kenya Medical Research Institute, Centre for Traditional Medicine & Drugs Research (KEMRI-CTMDR), of plants that have previously been assessed for antiplasmodial activity was used to select the study plants. From this database, six medicinal plants that had shown high *in vitro* antiplasmodial activity of IC_{50} < 10 µg/ml against chloroquine sensitive D6 strain of *P. falciparum* were selected.

3.2 Plant collection and treatment

The plant materials used had previously been collected from different parts of the country namely Kilifi and Kisumu Counties. The plants had been identified by a taxonomist prior to collection, allocated voucher specimen numbers and the voucher specimen deposited at the National Museum of Kenya, Nairobi.

3.3 Raw material handling

Plant materials were dried under a shade for a period of two weeks and milled using a Willy mill grinder. The dried powders were stored in 1kg manila paper bags and kept in a cool dry room until use.

3.4 Preparation of crude extracts for preliminary analysis

100g of each powdered material was soaked in 200ml 100% methanol for 48 hours. The resultant solution was decanted and filtered using Whatman filter paper 1(185mm). Solvent was removed under low pressure using a rotary vacuum evaporator, dried further at room temperature, weighed and the yield calculated and stored in glass bottles in the fridge at -20^{o}C until use. Water extracts were obtained as follows; powdered plant material (100g) was weighed into a beaker, and distilled water, enough to cover the material added. The beaker was heated in a water bath at 60-70^{o}C for about 1 hour and the mixture decanted after cooling. The solution was then filtered using Whatman filter paper 1(185mm) and transferred into a round-bottom flask. The flask was immersed in dry ice and the extract freeze-dried for about 48hr. The lyophilized extract was

weighed and kept in an airtight glass bottle in the freezer at -20^{o}C until use. The yield was calculated and recorded.

3.5 *In vitro* antiplasmodial screening of plant extracts

Erythrocytic stages of chloroquine resistant strain of *P. falciparum* (W2 strain from Indochina) that had been cryopreserved in liquid nitrogen were obtained from Malaria laboratories of KEMRI, Nairobi, Kenya.

The frozen parasites were thawed, washed and transferred to a nutrient medium, known as Complete medium with serum (CMS) and erythrocytes previously derived from an O+ blood group donor. Once the parasites had been revived, they were cultured and maintained using a method previously described by Trager and Jensen (1976).

The culture conditions required for optimum growth and maintenance of the malaria parasites included; maintaining a temperature of 37°C, in a 3% O_2, 5% CO_2 and 92% N_2 atmosphere, nutrient media containing Roswell Park Memorial Institute Medium 1640 (RPMI 1640), 25 mM N-Hydroxyethylpiperazine-N-2-ethanol sulfonic acid buffer (HEPES buffer), pH 7.4, supplemented with heat inactivated 10% human serum and human erythrocytes. The culture medium was replaced every 24 hours and sub-culturing carried out every 48hours in order to maintain parasitemia of 2-10% and a 6% haematocrit. Synchronization to obtain >80% of the ring stage of the parasite was achieved by serial treatment with 5% sorbitol. The parasitized culture was diluted with uninfected erythrocytes to the working parasitemia level of 0.4%.

Stock test solutions were prepared at a concentration of 1mg/ml in dimethyl sulphoxide (DMSO) and kept in the fridge at 4^{o}C until use. The working solutions were prepared by diluting the stock solutions accordingly in RPMI which also reduced the concentration of DMSO to <1%, a level not toxic to the parasite. Serial microdilution assay technique described by (Desjardins et al., 1979) was used. The experiment was conducted in a 96 well plate in duplicate; with 500µg/ml chloroquine serving as the positive control and parasitized erythrocytes without drug and non-parasitized erythrocytes serving as controls for normal growth and no growth respectively. The plates were incubated in a humidified incubator at 37°C in a 3% O_2, 5% CO_2 and 92% N_2 atmosphere for 48hours. Radio-labelled Hypoxanthine (0.5µCi in 25µL of culture medium) was then added to each well and the plates incubated further for 18 hours. Each plate was kept in a

refrigerator (4°C) to halt the reaction and to keep the cultures prior to cell harvesting. Cells were harvested with a BetaplateTM cell harvester (Wallac, Zurich, Switzerland), into glass fibre filters which were then dried in an incubator at 37°C for 24h after which radioactivity was determined, using BetaplateTM liquid scintillation counter (Wallac, MicroBeta TriLux) to ascertain the incorporation of radio-labelled hypoxanthine.

The results obtained were noted down as counts per minute (cpm) per well for each extract concentration. IC_{50} for each extract was calculated by logarithmic transformation of drug concentration and counts per minute using the formula by Sixsmith *et al,*. 1984.

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

Where Y_{50} is the cpm value midway between parasitisized and non-parasitisized control cultures and X_1, Y_1, X_2, and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints.

3.6 *In vitro* antiplasmodial activity in herb-herb interaction experiments

The method previously described by Canfield *et al*., (1995) was used with modification, where concentrations higher than the estimated IC_{50} values (between 20 to 50 times) were combined at a ratio of 50:50 of extracts A and B. Single and combined extracts were dispensed into 96 well microtitre plates (Fivelman *et al.*, 1999). The plates were then incubated and tested following the *in vitro* antiplasmodial assay method above (Desjardins *et al*. 1979, Le Bras & Deloron 1983).

IC_{50} values obtained for individual extracts and the combinations were used to determine the level of synergy according to Berenbaum (1978) using the formula below to determine the Sum of fractional inhibition concentration (SFIC).

$$SFIC = \frac{Ac}{Ae} + \frac{Bc}{Be}$$

where A_c and B_c are the IC_{50} for combined extracts and A_e and B_e are IC_{50} for the single extract. The results were interpreted as follows: SFIC <1 implies synergism, ≥1 but <2 imply additive interaction and ≥2 implies antagonism (Gupta *et al.*, 2002)

3.7 *In vivo* antiplasmodial evaluation

The protocol used was based on the 4-day suppressive test as described by Peters *et al*. (1975) where *P. berghei* infection of mice was used to assess the efficacy of a plant extract against the parasite. Mice were reared in the KEMRI animal house facility following OECD and KEMRI guidelines on laboratory animal use and care. Swiss albino mice, aged 6 weeks and of the same sex, weighing 20±2g were divided in groups of five and kept in 15 × 21 × 29 cm polypropylene cages in an air-conditioned animal room at 22-23 °C. The cages were bedded with wood shavings which were changed every two days and furnished with continuous-flow nipple watering devices. The mice were fed with pelleted mouse feed and given unlimited drinking water. The cages were designated as I, II etc and labeled with the details of each experiment as shown in Figure 9.

Figure 9: Labeled experimental cages with mice

P. berghei stabilates, previously kept at -80 °C were collected and thawed at room temperature. The parasitisized RBC's were reconstituted in phosphate buffered saline (PBS) and injected

intraperitoneally into naïve recipient passage mice, using 23G needle. Parasite growth was monitored by collecting blood samples from the tail vein punctured using 27G needles and a small drop of blood (50 µl) placed on a clean glass slide and spread to make a thin smear. The smears were fixed in methanol for about 2 minutes, stained in 10% Giemsa and observed under a microscope at X100 magnification. On achieving parasitemia of about 20%, the passage mice underwent cardiac puncture, where the mice are anaesthetized using CO_2 in a gas chamber and the thoracic cavity opened to draw blood from the ventricles. The blood drawn was diluted with PBS to achieve 2% parasitemia and 0.2ml of the blood injected intraperitoneally into each experimental mouse.

The four-day suppressive test was carried out as a preliminary screening of the herbal extracts. Each group comprising of three, 6-week-old mice of the same sex, were infected with 0.2ml parasitized blood containing approximately $2x10^7$ red blood cells of *P. berghei* on day zero of the test. The negative control group was given PBS solution while the positive control was given 10 mg/ml Artemether orally.

The plant test extracts were weighed and reconstituted as follows: they were solubilised in 10% Tween 80 and 3% DMSO for the methanol extracts and dissolved in distilled water for the water extracts to achieve a concentration of 250 mg and 500 mg per kg body weight. These were then administered orally once a day at a dose volume of 0.2ml per mouse using 22G stainless steel feeding cannula, 2 hr post infection and subsequently after 24, 48 and 72 hr respectively. On day 4 of the test, blood was drawn from the tail vein of each mouse, thin smear made, fixed with methanol and stained with 10% Giemsa. The slides were observed under a microscope at X100 magnification and parasitized and total RBC's determined as shown in Figure 10, from which percentage parasitemia value was calculated as described by Kalra *et al.*, 2006. The formula is as follows:

$$\% \text{ parasitemia} = \frac{\text{No. of parasitized RBC's}}{\text{Total No. of RBC's}} \times 100$$

Percentage Parasite growth suppression (PGS) or chemosuppresion was calculated using the formula as given by (Dikasso *et al.*, 2006):

$$\text{Percentage chemosuppression} = \frac{\text{Mean parasitaemia in the negative control group} - \text{Parasitaemia in the test group}}{\text{mean parasitaemia in the negative control group}} \times 100$$

Survival duration of the mice was noted and those that got severely ill were euthanized using the following humane endpoints for severe malaria: respiratory distress, convulsions, severe anaemia, severe pain or distress. At the conclusion of the experiment, all the surviving mice were euthanized using CO_2, the carcasses put in biohazard bags and incinerated.

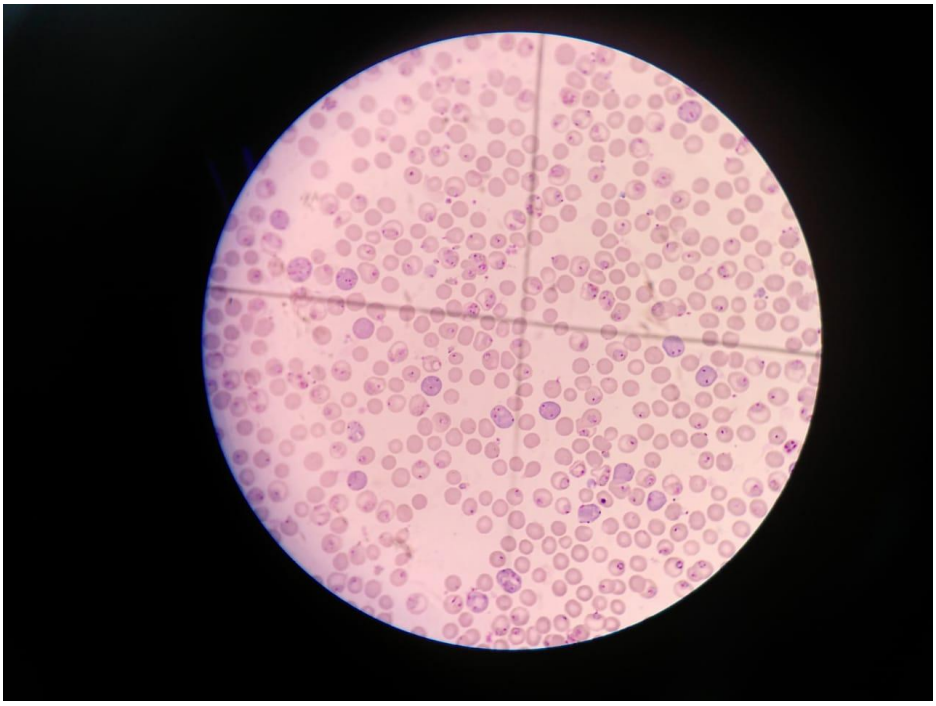

Figure 10: View of parasitized and non-parasitized RBC under a microscope

3.8. Cytotoxicity studies

The Tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) based assay method (MTT) was used to determine cell viability due to its simplicity and effectiveness. Vero cells are kidney-derived adherent cells from the African green monkey used in cell biology research. These cells are stored at -80 ^{o}C or in liquid Nitrogen. From storage, the cells were thawed at 37^{o}C in a water bath after which the suspension was transferred to 15ml conical tube containing 10ml of Eagle's Minimum Essential Medium (DMEM) supplemented with 10% foetal bovine serum (FBS). The cells were pelleted by centrifugation at 200 revolutions per

minute for 5minutes at room temperature. After discarding the supernatant solution, the cells were resuspended in 10ml DMEM supplemented with 10% FBS and transferred to tissue culture flask and incubated at 37^{o}C in 5% CO_2. The medium was changed every 3-4 days until the cells reached a >90% confluent monolayer. The Vero cells were seeded in a 24-well flat bottomed microtitre plate at a density of 2 x 10^5 cells per well and allowed to adhere for 24 hours at 37^{o}C in 5% CO_2. The culture medium was then replaced by fresh DMEM medium containing extracts of the two plants that had shown the best *in vivo* antiplasmodial efficacy at 1000 ug/ml and further incubated for 48hours under the same conditions. 10 uL of MTT working solution was added to each well and the plates further incubated for 4 hours. The medium was then aspirated e formed formazan crystals solubilized by addition of 50 uL of DMSO per well for 30 minutes at 37 ^{o}C. The intensity of the dissolved formazan (purple colour) was quantified using a scanning multiwell spectrophotometer at 540 nm and 720 nm as shown in Figure 11. To calculate the percentage of viable cells, the formula below was used.

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

A dose-response curve was plotted from which CC_{50} (concentration that causes death of 50% of cells) was estimated. Selectivity index was then calculated as follows: $SI = CC_{50}/IC_{50}$

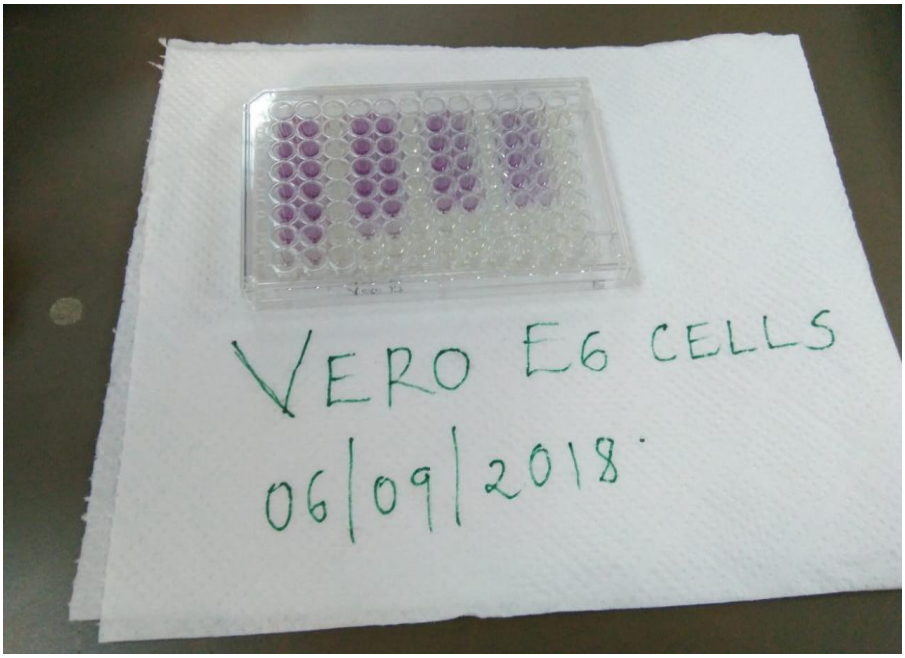

Figure 11: A Vero 199 cell cytotoxicity assay showing wells with purple coloured formazan with cells present and controls with no cells present.

3.9 Acute oral toxicity study

This was done in accordance with Organization for Economic Co-operation and Development (OECD) guidelines for testing of chemicals 420. (OECD, 2001)

Female albino mice of between 8 to 12 weeks, weighing 20g±2 were selected and grouped in three's. The mice were weighed individually and marked before the test. Prior to extract administration, food was withheld for 3-4 hours and 1-2 hours post administration but water was availed throughout. The mice were given orally, fixed doses of 1000, 1500 and 2000 mg/kg body weight of the methanolic extracts of *S. pinnata* and *T.asiatica*. Observations on the mice were done at least once within the first 30 minutes and then periodically within 24 hours. After 24 hours, they were checked for signs of toxicity which included: death, changes in skin and fur, eyes, mucus membranes, autonomic and central nervous systems such as tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. The number of deaths was noted and LD_{50}values for each extract calculated.

3.10 Data management

Records of all laboratory activities were kept in a notebook. For reproducibility of results, experiments were carried out in duplicate or triplicate where applicable. *In vivo* antiplasmodial activity was determined by the percentage suppression of parasitemia and survival rates at each dose level with reference to the control.

3.11 Ethical considerations

The study was carried with the permission granted by KNH/UON Ethics Review Committee (Ref: P680/009/2018), Animal Care and Use Committee (ACUC), and KEMRI Scientific and Ethics Review Unit (SERU/SSC/2928). The research followed the internationally prescribed W.H.O and KEMRI guidelines on animal use and care. The work was carried out following the principles of Good laboratory practice at KEMRI-CTMDR laboratories which are GLP compliant.

3.12 Biosafety considerations

Personal protective clothing was donned at all times during the animal experiments. Carcasses of infected animals were disposed of by incineration.

CHAPTER 4
4.0 RESULTS AND DISCUSION

4.1 Selected Plants

Plants studied were collected from Kilifi and Kisumu counties. They were selected from a database available at KEMRI-CTMDR and coded as shown in the Table 1 below. These plants had been reported to have high antiplasmodial activity *in vitro* (<10 µg/ml) against Chloroquine sensitive *P. falciparum*. They had previously been harvested, dried, milled and stored. The plant part and extract used are shown in the Table 1 below.

Table 1: Plant description and details

Plant	**Voucher specimen number**	**Code**	**Origin**	**Plant part**	**Extract**
Carissa edulis	JO/JG/IRG/028/2016	CE001	Malindi	Root bark	water
Zanthoxylum chalybeum	JO/JG/IRG/001/2016	ZC002	Malindi	Leaves	water
Flueggea virosa	JO/JG/IRG/027/2016	FV003	Malindi	Leaves	methanol
Schkuria pinnata	JO/JG/IRG/029/2016	SP004	Malindi	Whole plant	methanol
Boscia angustifolia	JO/JG/IRG/009/2016	BA005	Malindi	Stem bark	methanol
Toddalia asiatica	JO/JG/IRG/015/2016	TA006	Kisumu	Fruits	methanol

4.2 *In vitro* results

Activity criteria in the *in vitro* assay for unpurified extracts were classified as follows: high at IC_{50} ≤10 µg/ml, moderate at 10–50 µg/ml, low at 50–10 µg/ml and inactive at >100 µg/ml (Gathirwa *et al* 2008). Following this criteria, the results obtained were summarized as follows for both single and combined extracts in Table 2 and 3, respectively.

4.2.1 Single extracts

In vitro results of single extracts showed that the most active extract against the chloroquine resistant W2 strain of *P. falciparum* was SP004 having an IC_{50} of 10.8 µg/ml. Moderate activity was exhibited by ZC002, FV003 and BA005 while TA006 had low activity. CE001 was found to be inactive.

Table 2: Antiplasmodial activity of single extracts

Extract	CE001	ZC002	FV003	SP004	BA005	TA006
IC_{50} µg/ml	>100	37.7±0.959	19.028±0.392	10.808±0.408	20.257±0.770	65.632±0.876

4.2.2 Combination of extracts

When different extracts were combined at equal ratios of 50:50, different scenarios were observed. The most active combination were ZC002/FV003, ZC002/SP004, ZC002/BA005, ZC002/TA006 having IC_{50}'s <10 µg/ml. Those that were categorized as having moderate activity were CE001/BA005, FV003/SP004, SP004/BA005, CE001/ZC002, CE001/FV003, CE001/SP004, FV003/BA005, SP004/TA006 and FV003/TA006 having IC_{50}'s of between 10 and 50 µg/ml CE001/TA006 and BA005/TA006 had low activity.

Table 3 summarizes the Sum of fractional inhibition concentration (SFIC) which was used to interpret the results obtained on combination as follows: SFIC <1 implies synergism, ≥1 but <2 imply additive interaction and ≥2 implies antagonism (Gupta *et al.*, 2002).

Antagonistic combinations included: CE001/BA005, CE001/TA006, FV003/SP004, SP004/BA005 and BA005/TA006. Combinations that were additive included: CE001/ZC002, CE001/FV003, CE001/SP004, FV003/BA005 and SP004/TA006. Combinations that were synergistic were: ZC002/FV003, ZC002/SP004, ZC002/BA005, ZC002/TA006 and FV003/TA006.

The preliminary *in vitro* results were used to determine combinations that were to be subjected to *in vivo* tests. This selection therefore omitted combinations that were antagonistic.

Table 3: Antiplasmodial activity of combined extracts

Extracts	IC_{50} µg/ml	SFIC	Comment
CE001/ZC002	41.985	1.53371	additive
CE001/FV003	16.414	1.02676	additive
CE001/SP004	12.665	1.29847	additive
CE001/BA005	46.968	2.78829	antagonistic
CE001/TA006	85.519	2.1582	antagonistic
ZC002/FV003	5.533	0.4376	synergistic
ZC002/SP004	3.23	0.38456	synergistic
ZC002/BA005	9.409	0.71414	synergistic
ZC002/TA006	9.244	0.38613	synergistic
FV003/SP004	39.26	5.69577	antagonistic
FV003/BA005	12.254	1.24893	additive
FV003/TA006	11.392	0.77227	synergistic
SP004/BA005	11.425	6.88314	antagonistic
SP004/TA006	13.471	1.45164	additive
BA005/TA006	59.984	3.87509	antagonistic
Chloroquine phosphate	124.04 ng/ml		

4.3 *In vivo* results

Both single and combined extracts were tested *in vivo* to enable comparison of their efficacy. Percentage parasite growth suppression was calculated for each extract at different doses from the percentage parasitemia observed in each mouse set compared to the negative control.

4.3.1 Single extract

Single extracts elicited mixed results at different doses as shown in Table 4. Only SP004 and TA006 showed considerable parasite growth suppression at 250 mg/kg. At 500 mg/kg however, only TA006, showed significant parasite growth suppression. SP004 showed no parasite growth suppression at 500 mg/kg. At 250 mg/kg CE001, ZC002, FV003 showed no activity at all, with the test mice experiencing higher levels of parasitemia than the negative control. At 500 mg/kg dose, there was still no significant change in inactivity of CE001, ZC002, FV003 and BA005 which exhibited negative suppression in the mice. Seeing that SP004 seemed to cause toxicity at higher dose of 500 mg/kg, the tests were repeated at 100 mg/kg and 250 mg/kg where parasite

0growth suppression of 54.6 and 67.4% were achieved. This indicated that the optimal dose of SP004 was below 500 mg/kg.

Table 4: Percentage parasite growth suppression of single extracts

Extract	250 mg/kg	500 mg/kg	100 mg/kg
CE001	-64.2	1.5	ND
ZC002	-19.0	-20.3	ND
FV003	-43.6	3.7	ND
SP004	66.3±1.1	-3.8	54.6
BA005	12.7	-11	ND
TA006	51.6	58.9	ND

Key: ND-Not done

4.3.2 Combined extracts

As can be seen in Table 5, at a dose of 250 mg/kg, all the combined extracts showed negative parasite growth suppression except CE001/SP004 which suppressed parasite growth slightly by 20%. At 500 mg/kg however, there was significant increase in the percentage parasite growth suppression. The combination that showed the most significant parasite growth suppression was SP004/TA006 at a dose of 500 mg/kg giving a percentage parasite growth suppression of 81.3 which was comparable to that of the positive control. This combination was subjected to further testing at a dose of 650 mg/kg and a ratio of 1:4 as shown on Table 5. The combination of SP004/TA006 gave a parasite growth suppression of 85.5% which was compared to the positive control of Artemether 10 mg/kg with a parasite growth suppression of 88%.

Table 5: Percentage parasite growth suppression results of combined extracts

Extract	250 mg/kg	500 mg/kg	125/500 mg/kg
CE001/SP004	20.0	66.3	ND
ZC002/FV003	-12.2	28.9	ND
ZC002/SP004	-30	43.2	ND
ZC002/BA005	-32.9	24.2	ND
ZC002/TA006	-24.2	49.8	ND
FV003/BA005	-24.2	41.5	ND
FV003/TA006	1.7	46.0	ND
SP004/BA005	-33.6	40.5	ND
SP004/TA006	-61.9	81.3	85.5
Artemether at standard dose of 10 mg/kg body weight	89.3	87.2	88

4.3.3 Combination of SP004/TA006 at different doses and ratio of 1:4

From the results obtained in section 4.3.2 above, the combination of SP004/TA006 at a combination ratio of 1:4 proved to have the best parasite suppression ability and was tested further at different doses (Table 6). At the highest concentration of 1000 mg/kg, there was higher parasitemia compared to the negative control. Mice administered this dose had the least survival at day 7. Lower doses showed increased survival rate on day 7 and better parasite growth suppression.

Table 6: *In vivo* results of combination of *S. pinnata* and *T. asiatica* at different doses

Dose	% parasetemia	% Parasite growth suppression	%Mice survival on day 7
Artemether (10 mg/kg)	10	75.2	100
PBS(negative control)	42.1	-	60
1000 mg/kg	69.4	-65	20
750 mg/kg	15.3	63.6	40
500 mg/kg	12.4	70.6	60
250 mg/kg	5.99	85.8	100

4.4 Cytotoxicity studies

Having selected SP004 and TA006 as the two extracts that gave the best parasite growth suppression, the two were subjected to cytotoxicity tests with the following results; SP004 had a CC_{50} of 102.93±5.96 µg/ml while TA006 had CC_{50}>1000 µg/ml. Their selectivity indices were found to be 9.52 and 15.24 respectively indicating good therapeutic potential due to their safety margins. Selectivity index greater than 4 is generally considered as safe value for antimalarial extracts (Valdés *et al*., 2010).

4.5 Acute toxicity results

The extracts of SP004 and TA006 were subjected to acute toxicity test which showed that both extracts had LD_{50} values >2000 mg/kg with no observable changes in mice disposition at that dose.

4.6 Discussion of Results

From the confirmatory *in vitro* tests of single extracts, it was found out that water extract of the root back of *C. edulis* and the methanol extract of the fruit of *T.asiatica* did not have activity against chloroquine resistant *P. falciparum*. *C. edulis* did not also have any activity in the *in vivo* tests. *T. asiatica* however had moderate activity against *P. berghei* validating its use in traditional medicine against malaria. Water extract of the leaves of *Z. chalybeum* exhibited moderate activity in the *in vitro* test but no activity *in vivo*. The low or moderate activity of *T. asiatica* and *Z. chalybeum* against chloroquine resistant *P. falciparum* can be hypothesized to be due to the nature of phytochemicals present. Alkaloids are a major classe of phytochemicals that exhibit antiplasmodial activity. *T. asiatica* and *Z. chalybeum* belong to the Rutacea family which is known to contain quinoline alkaloids. Chloroquine is similarly a quinoline alkaloid, hence would explain the relatively low activity against the chloroquine resistant *P. falciparum*. S. pinnata showed both high activity against chloroquine resistant *P. falciparum* and *P. berghei*. It belongs to Astraceae family and is known to contain sesquiterpene lactones just as *Artemesia annua* which has high antiplasmodial activity (Kimani *et al.,* 2017). This result confirms those from different studies that showed high activity of *S. pinnata* against both chloroquine sensitive and resistant *P. falciparum* (Muthaura *et al*., 2007). *B. angustifolia* and *F. virosa* showed moderate activity against chloroquine resistant *P. falciparum* and no or negligible activity *in vivo.*

Combination of extracts *in vitro* resulted in synergy, addition or antagonism, factors which must be considered in making a selection of extracts to combine. *Z. chalybeum* combinations were synergistic with very high activity. However, these combinations only showed moderate *in vivo* efficacy at 500 mg/kg warranting further testing. This could be due to the pharmacokinetic principles that affect the disposition of the active ingredients in the extracts.

From *in vivo* studies, moderate activity of combined extracts was obtained at a dose of 500 mg/kg. Lower doses of 250 mg/kg exhibited worse parasite control implying interference with immunity of the mice. *T. asiatica* extract showed inactivity *in vitro* but moderate activity *in vivo*. A possible explanation could be that some of its active ingredients are prodrugs.

A combination of *T. asiatica* and *S. pinnata* exhibited synergism *in vitro* and high parasite growth suppression *in vivo*, making it the best combination for pre-formulation studies. In terms of toxicity, it was noted that *S. pinnata* was more toxic than *T. asiatica* as indicated by the reduced chemo-suppression at higher doses of 500 mg/kg and lower cytotoxicity index.

4.7 CONCLUSION AND RECOMMENDATION

The results obtained from both *in vitro* and *in vivo* studies validate the use of these selected plants in traditional management of malaria. Combination studies indicate that there can be different results obtained, including synergy, antagonism and potentiation. It is also evident that the doses used elicit different results. Certain extracts at higher doses were more toxic leading to higher parasetemia than the negative control. Paradoxically, in the *in vivo* tests, certain extracts showed higher levels at parasetemia at 250 mg/kg but better activity at 500 mg/kg. All extracts exhibited better parasite growth suppression when in combination proving that combining different extracts can improve efficacy against Plasmodium parasite. Of note is that *in vitro* activity may not translate to equal *in vivo* efficacy. This is evidenced by the high *in vitro* activity noted with *Z. chalybeum* combinations but low parasite growth suppression on *in vivo* tests.

A combination of *T. asiatica* and *S. pinnata* methanol extracts showed promising parasite growth suppression against *P. berghei* that was comparable with Artemether. It had additive effect against Chloroquine resistant *P. falciparum* from the *in vitro* test. Based on the results obtained, it is recommended that further studies be carried out of *T. asiatica* and *S. pinnata* extracts to find optimum efficacious dose combinations that can be formulated into a malaria drug seeing that

combination of the two is more effective than the use of a single extract. Cytotoxicity of the combination should be carried out and compared to that of the single extracts. Furthermore, chronic toxicity studies on the two extracts should be undertaken to determine their long term toxicity. Since the plant part selected was the fruit of *T. asiatica*, which from previous studies had elicited the best activity, it would be of interest to subject other parts such as the leaves to a similar study (Orwa *et al.*, 2013). Phytochemical screening of the two extracts should be conducted to determine the compounds responsible for activity.

References

Agbodeka, K., Gbekley, H., Karou, S., Anani, K., Agbonon, A., Tchacondo, T., Batawila, K., Simpore, J. and Gbeassor, M. (2016) 'Ethnobotanical study of medicinal plants used for the treatment of malaria in the plateau region, Togo', *Pharmacognosy Research*, 8(5), p. 12. doi: 10.4103/0974-8490.178646.

Anywar, G., van't Klooster, C. I. E. A., Byamukama, R., Willcox, M., Nalumansi, P. A., de Jong, J., Rwaburindori, P. and Kiremire, B. T. (2016) 'Medicinal plants used in the treatment and prevention of malaria in Cegere sub-county, Northern Uganda', *Ethnobotany Research and Applications*, 14(January), pp. 505–516. doi: 10.17348/era.14.0.505-516.

Balunas, M. J. and Kinghorn, A. D. (2005) 'Drug discovery from medicinal plants', 78, pp. 431–441. doi: 10.1016/j.lfs.2005.09.012.

Berenbaum MC., (1978) 'A method for testing for synergy with any number of agents'. *Journal of Infectious Diseases*, 137: 122-130.

Bloland, P. B., Ettling, M. and Meek, S. (2000) 'Combination therapy for malaria in Africa : hype or hope ?' 78 (12).

Burkill, H.M., 1985. The useful plants of West Tropical Africa. 2nd Edition. Volume 1, Families A–D. Royal Botanic Gardens, Kew, Richmond, United Kingdom. 960 pp.

Canfield, C.J., Pudney, M., Gutteridge, W.E., (1995) 'Interactions of atovaquone with other antimalarial drugs against *Plasmodium falciparum in vitro'*. *Experimental Parasitology*, 80: 373–381.

Desjardins RE., Canfield CJ., Haynes JD., Chulay JD., (1979) 'Quantitative assessment of antimalarial activity *in vitro* by semi-automated microdilution technique'. *Antimicrobial Agents and Chemotherapy*16: 710-718.

Dharani, N., Rukunga, G., Yeneser, A., Mbora, A., Mwaura, L., Dawson, I. and Jamnadass, R. (2010) *Common Antimalarial Trees and Shrubs of East Africa, World Agroforestry Centre*.

Dikasso D., Mekonnen E., Debella A., (2006) '*In vivo* antimalarial activity of hydroalcoholic extracts from *Asparagus africanus* Lam. in mice infected with *Plasmodium berghei'*. *Ethiop J Health Dev*.; 20:112–118

Evans WC.,(2009) Trease and Evans' Pharmacognosy E-Book. Elsevier Health Sciences.

Fivelman, Q. L., Walden, J. C., Smith, P. J., Folb, P. I. and Barnes, K. I. (1999) '* OF * Ow', pp. 429–432.

Gallup, J. L. and Sachs, J. D. (2001) 'THE ECONOMIC BURDEN OF MALARIA', 64, pp. 85–96.

Gathirwa, J. W., Rukunga, G. M., Njagi, E. N. M., Omar, S. A., Mwitari, P. G., Guantai, A. N., Tolo, F. M., Kimani, C. W., Muthaura, C. N., Kirira, P. G., Ndunda, T. N., Amalemba, G., Mungai, G. M. and Ndiege, I. O. (2008) 'The *in vitro* anti-plasmodial and *in vivo* anti-malarial efficacy of combinations of some medicinal plants used traditionally for treatment of malaria by the Meru community in Kenya', *Journal of Ethnopharmacology*, 115(2), pp. 223–231. doi: 10.1016/j.jep.2007.09.021.

Graz, B., Willcox, M. L., Diakite, C., Falquet, J., Dackuo, F., Sidibe, O., Giani, S. and Diallo, D. (2010) '*Argemone mexicana* decoction versus artesunate-amodiaquine for the management of malaria in Mali: policy and public-health implications', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 104(1), pp. 33–41. doi: 10.1016/j.trstmh.2009.07.005.

Gupta, S., Thapar, M. M., Mariga, S. T., Wernsdorfer, W. H. and Björkman, A. (2002) '*Plasmodium falciparum*: *In vitro* interactions of artemisinin with amodiaquine, pyronaridine, and chloroquine', *Experimental Parasitology*, 100(1): 28–35. doi: 10.1006/expr.2001.4659.

Iii, P. and Africa, S. (2014) 'RELEASE Malaria vaccine candidate has demonstrated efficacy over 3-4 years of follow-up', pp. 6–8.

Kalra B.S., Chawla S., Gupta P., Valecha N (2006) 'Screening of antimalarial drugs'. *Ind J Pharmacol*.; 38:5–12

Kaou, A. M., Mahiou-Leddet, V., Hutter, S., Aïnouddine, S., Hassani, S., Yahaya, I., Azas, N. and Ollivier, E. (2008) 'Antimalarial activity of crude extracts from nine African medicinal plants', *Journal of Ethnopharmacology*, 116(1): 74–83. doi: 10.1016/j.jep.2007.11.001.

Kebenei, J. S., Ndalut, P. K. and Sabah, A. O. (2011) Anti-plasmodial activity of nortrachelogenin from the root bark of *Carissa edulis*', *International Journal of Applied Research in Natural Products*, 4(3): 1–5.

Kimani, Njogu & C. Matasyoh, Josphat & Kaiser, Marcel & Brun, Reto & Schmidt, Thomas. (2017). Antiprotozoal Sesquiterpene Lactones and Other Constituents from Tarchonanthus camphoratus and Schkuhria pinnata. Journal of Natural Products. 81. 10.1021/acs.jnatprod.7b00747.

Klayman, D. (1985) 'Qinghaosu (artemisinin): an antimalarial drug from China', *Science*, 228(4703), 1049–1055. doi: 10.1126/science.3887571.

Koch A., Tamez, P., Pezzuto, J., Soejarto D. (2005) 'Evaluation of plants used for anti-malarial treatment by the Maasai of Kenya'. *Journal of Ethnopharmacology*, 101: 95-99.

Kokwaro, J. O., 1976. Medicinal plants of east Africa. East African Literature Bureau, Nairobi, Kenya, pp. 1-8, 25-26.

Kokwaro JO (2008). Medicinal Plants of East Africa. Kenya Literature Bureau, Nairobi, Kenya.

Le Bras J., Deloron P. (1983) 'In vitro study of drug sensitivity of *Plasmodium falciparum*: An evaluation of a new semi-microtest.' *Am. J. Trop. Med. Hyg*., 32: 447-51.

Mahomoodally, F.M., (2013) 'Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants, *Evidence-Based Complementary and Alternative Medicine'*. 2013: 1-14. https://doi.org/10.1155/2013/617459.

Mesia, K., Cimanga, R. K., Dhooghe, L., Cos, P., Apers, S., Totté, J., Tona, G. L., Pieters, L., Vlietinck, A. J. and Maes, L. (2010) Antimalarial activity and toxicity evaluation of a quantified *Nauclea pobeguinii* extract, *Journal of Ethnopharmacology*, 131(1): 10–16. doi: 10.1016/j.jep.2010.05.008.

Mesia, K., Tona, L., Mampunza, M., Ntamabyaliro, N., Muanda, T., Muyembe, T., Musuamba, T., Mets, T., Cimanga, K., Totté, J., Pieters, L. and Vlietinck, A. (2012) 'Antimalarial Efficacy of a Quantified Extract of *Nauclea pobeguinii* Stem Bark in Human Adult Volunteers with

Diagnosed Uncomplicated falciparum Malaria. Part 2: A Clinical Phase IIB Trial', *Planta Medica*, 78(9): 853–860. doi: 10.1055/s-0031-1298488.

Mills, S., Bone, K., (2000) Principles and Practice of Phytotherapy. Edinburgh: Churchill Livingstone.

Muthaura, C. N., Rukunga, G. M., Chhabra, S. C., Omar, S. A., Guantai, A. N., Gathirwa, J. W., Tolo, F. M., Mwitari, P. G., Keter, L. K., Kirira, P. G., Kimani, C. W., Mungai, G. M. and Njagi, E. N. M. (2007) 'Antimalarial activity of some plants traditionally used in treatment of malaria in Kwale district of Kenya', *Journal of Ethnopharmacology*, 112(3), pp. 545–551. doi: 10.1016/j.jep.2007.04.018.

Muthaura, C.N., Rukunga, G.M., Chhabra, S.C., Omar, S.A., Guantai, A.N., Gathirwa, J.W., Tolo, F.M., Mwitari, P.G., Keter, L.K., Kirira, P.G., Kimani, C.W., Mungai, G.M & Njagi, E.N.M. 2007. Antimalarial Activity of Some Plants Traditionally used in Meru district of Kenya. Phytother. Res., 21: 860–867.

Ngarivhume, T., Van'T Klooster, C. I. E. A., De Jong, J. T. V. M. and Van Der Westhuizen, J. H. (2015) 'Medicinal plants used by traditional healers for the treatment of malaria in the Chipinge district in Zimbabwe', *Journal of Ethnopharmacology*. Elsevier, 159: 224–237. doi: 10.1016/j.jep.2014.11.011.

Nguta, J. M., Mbaria, J. M., Gathumbi, P. K., Gakuya, D., Kabasa, J. D. and Kiama, S. G. (2011) 'Ethnodiagnostic Skills of the Digo Community for Malaria: A Lead to Traditional Bioprospecting', *Frontiers in Pharmacology*, 2. doi: 10.3389/fphar.2011.00030.

Odediran, S.A.; Elujoba, A.A.; Adebajo, C.A. (2014) 'Influence of formulation ratio of the plant components on the antimalarial properties of MAMA decoction'. *Parasitol. Res*., 113, 1977–1984.

Odugbemi T.O.; Akinsulire, O.R.; Aibinu, I.E.; Fabeku P.O. 'Medicinal plants useful for malaria therapy in Okeigbo, Ondo State, Southwest Nigeria'

African Journal of Traditional Complementary and Alternative Medicine, 4 (2007), pp. 191-198

Ogwang, Patrick & Ralph, Tumusiime & Agwaya, Moses & Gerosome, Mugisha & Kyeyune, Grace & Badru, Galiwango & Waako, Paul. (2008). Repeat-dose effects of Zanthoxylum chalybeum root bark extract: A traditional medicinal plant used for various diseases in Uganda. Afr J Pharm Pharmacol. 2.

Organization of Economic Co-operation and Development. The OECD guideline for testing of chemical: 420 Acute Oral Toxicity–Fixed Dose Method. Paris France; 2001a. Available at http://www.oecd-ilibrary.org/environment/test-no-420-acute-oral-toxicity-fixed-dose-procedure_9789264070943-en.

Orwa C., Mutua A., Kindt R., Jamnadass R., Anthony S., (2009). Agroforestree Database:a tree reference and selection guide version 4.0 http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp)

Orwa, J. A., Jondiko, I. J. O., Minja, R. J. A. and Bekunda, M. (2008) 'The use of *Toddalia asiatica* (L) Lam. (Rutaceae) in traditional medicine practice in East Africa', *Journal of Ethnopharmacology*, 115(2): 257–262. doi: 10.1016/j.jep.2007.09.024.

Orwa, J. A., Ngeny, L., Mwikwabe, N. M., Ondicho, J. and Jondiko, I. J. O. (2013) 'Antimalarial and safety evaluation of extracts from *Toddalia asiatica* (L) Lam. (Rutaceae)', *Journal of Ethnopharmacology*, 145(2): 587–590. doi: 10.1016/j.jep.2012.11.034.

Payne, D. (1987) 'Spread of chloroquine resistance in *Plasmodium falciparum*', *Parasitology Today*, 3(8): 241–246. doi: 10.1016/0169-4758(87)90147-5.

Peters W., Portus JH., Robinson BL.(1975) 'The chemotherapy of rodent malaria XXII. The value of drug-resistant strains of *Plasmodium berghei* in screening for schizontocidal activity'. *Ann Trop Med Parasitol*, 69: 155-171.

Rasoanaivo P, Wright CW, Willcox ML, Gilbert B (2011) 'Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions'. *Malaria Journal*; 10(Suppl 1):S4. doi:10.1186/1475-2875-10-S1-S4.

Rukunga, G. M., Gathirwa, J. W., Omar, S. A., Muregi, F. W., Muthaura, C. N., Kirira, P. G., Mungai, G. M. and Kofi-Tsekpo, W. M., (2009) 'Anti-plasmodial activity of the extracts of some Kenyan medicinal plants', *Journal of Ethnopharmacology*, 121(2): 282–285. doi: 10.1016/j.jep.2008.10.033.

Singh, S. V., Manhas, A., Kumar, Y., Mishra, S., Shanker, K., Khan, F., Srivastava, K. and Pal, A. (2017) 'Antimalarial activity and safety assessment of Flueggea virosa leaves and its major constituent with special emphasis on their mode of action', *Biomedicine & Pharmacotherapy*, 89: 761–771. doi: 10.1016/j.biopha.2017.02.056.

Sixsmith DG, Watkins WM, Chulay JD, Spencer HC (1984) Invitro anti-malarial activity of tetrahydrofoliate dehydrogenase inhibitors. Am J Trop Med Hyg 33:772–776

Subregion, G. M. (2014) 'Status report on artemisinin resistance', *1. Subregion GM. Status report on artemisinin resistance. 2014;(January):1–7.*, 13(January), pp. 1–7. doi: 10.1017/CBO9781107415324.004.

Tabuti, J.R.S., (2007). *Flueggea virosa* (Roxb. ex Willd.) Voigt. [Internet] Record from PROTA4U. Schmelzer, G.H. & Gurib-Fakim, A. (Editors). PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands. <http://www.prota4u.org/search.asp>.

Trager, W., Jensen, J.B., (1976) 'Human malaria parasites in continuous culture'. *Science* **193**: 673–675.

Valdés, Aymé Fernández-Calienes, Martínez, Judith Mendiola, Lizama, Ramón Scull, Gaitén, Yamilet Gutiérrez, Rodríguez, Deyanira Acuña, & Payrol, Juan Abreu. (2010). In vitro antimalarial activity and cytotoxicity of some selected cuban medicinal plants. *Revista do Instituto de Medicina Tropical de São Paulo*, *52*(4), 197-201. https://dx.doi.org/10.1590/S0036-46652010000400006

Willcox, M. L. and Bodeker, G. (2004) 'Traditional herbal medicines for malaria', *BMJ*, 329(7475):1156–1159. doi: 10.1136/bmj.329.7475.1156.

Willcox, Merlin (2004). *Traditional Medicinal Plants and Malaria*. CRC Press. p. 231. *ISBN 9780203502327*.

WHO (2003) 'A report on Traditional medicines', *Fifty-Sixth World Health Assembly*, (March), pp. 14–17.

WHO (2006). ‘WHO briefing on malaria treatment guidelines and artemisinin monotherapies’

WHO (World Health Organization) (2011) The World Traditional Medicines Situation, in Traditional medicines: Global Situation, Issues and Challenges. Geneva 3:1–14.

WHO. 2013. *WHO Traditional Medicine Strategy 2014-2023*. Geneva: World Health Organization.

WHO (2016) *World Malaria Report, World Health Organization*. doi: 10.4135/9781452276151.n221.

World Health Organization (2018) ‘Artemisinin resistance and artemisinin-based combination therapy efficacy’, *Who*, (August), p. 10. Available at: https://www.who.int/malaria/publications/atoz/artemisinin-resistance-august2018/en/.

Ya’u, J., Yaro, A.H., Abubakar, M.S., Anuka, J.A and Hussaini, I.M., (2008) ‘Anticonvulsant activity of *Carissa edulis* (vahl) (Apocynaceae) root bark extract’. *Journal of Ethnopharmacology*, **120** (2): 255-8