ANALYSIS OF PHYTOCHEMICALS AND ANTI-PLASMODIAL ACTIVITIES OF EXTRACTS FROM *HARRISONIA ABYSSINICA, LEUCAS CALOSTACHYS* AND *RUBIA CORDIFOLIA* AGAINST *PLASMODIUM FALCIPARUM*

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF MASTER OF SCIENCE (APPLIED PARASITOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

DECEMBER, 2018

DECLARATION

I, Magara Jeremiah, declare that this thesis is my original work and has not been presented in any other institution for a degree or any other award.

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Supervisors' Approval

We confirm that the work reported in this thesis was carried out by the candidate under our supervision as the university supervisors.

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DEDICATION

I dedicate this work to my wife Ann Monchari and sons Newton Orenge and Brighton Magara for their patience and inspiration.

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ACRONYMS AND ABBREVIATIONS

ACD	Acid-Citrate-Dextrose
ACTs	Artemisinin-Combined Therapies
AL	Artemether- Lumefantrine
AQ	Amodiaquine
ANOVA	Analysis of Variance
AQ+AS	Amodiaquine and Artesunate
BCA	Biological Control Agents
BOC	British Oxygen Company
CAM	Complementally alternative medicine
CSP	Circumsporozoite Protein
CQ	Chloroquine
DHFR	Dihydrofolate Reductase
DMSO	Dimethylsulphoxide
DNA	DeoxyRibonucleic acid
DV	Digestive Vacuole
EM	Environmental Management
HEPES	2-hydroxyethlpiperazine-N-2ethanesulfonic acid
IC_{50}	Drug concentration killing 50% of test organisms *in vitro*
IRS	Indoor Residual Spraying
IGR	Insect Growth Regulators

IPR	Institute of Primate Research
KEMRI	Kenya Medical Research Institute
IGR	Insect Growth Regulators
LLINs	Long Lasting Insecticidal Nets
NMK	National Museums of Kenya
PMI	Presidents Malaria Initiative
P.C.V	Packed Cell Volume
RBC	Red Blood Cells
rpm	Revolutions Per Minute
PfCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
PfMDR1	*Plasmodium falciparum* Multidrug Resistance Protein 1
RPMI	Rosewell Park Memorial Institute Medium.
RES	Reticuloendothelia System
SSA	Sub Saharan Africa
STI	Sexually Tramited Infections
TID	Department of Tropical and Infectious Diseases
WHO	World Health Organization
W2	Chloroquine resistant *Plasmodium falciparum* strain
3D7	Chloroquine sensitive *Plasmodium falciparum* strain
K39	Chloroquine sensitive *P. falciparum* strain

ABSTRACT

Malaria is the commonest parasitic disease that continues to cause considerable number of deaths despite the fact that it is treatable and preventable. In 2016, nearly 3.2 billion persons were at risk of malaria and a total of 216 million cases occurred occasioning deaths of approximately 445 thousand people worldwide with majority of the affected being children below five years of age. These numbers are high for a disease which is treatable and preventable. *Plasmodium falciparum*, the deadliest human malaria parasite has become resistant to nearly all antimalarials previously used and its drug resistant strains have shown rapid extension thus complicating the fight against malaria. As such there is need for continuous search for new medicines. Studies on plants traditionally used for the treatment of malaria presents one of the most viable strategies of developing new and effective antimalarial drugs. This study evaluated the *in vitro* effects of crude extracts obtained from *Harrisonia abyssinica* Oliv., *Leucas calostachys* Oliv. and *Rubia cordifolia* L. frequently used in the herbal management of malaria and other infections among the Maasai community in Transmara West sub-county. Aqueous, methanol and hexane extracts were assessed against two *Plasmodium falciparum* strains namely, *Plasmodium falciparum* W2, (Chloroquine resistant) and *Plasmodium falciparum* 3D7, Chloroquine sensitive. The parasite strains were cultured in malaria laboratory at the Institute of Primate Research (IPR). The *in vitro* effects of the extracts on the parasite strains were evaluated in 12x8 flat bottom wells and sterile microtitre plates. All bioassays were performed in triplicate at eight concentrations ranging between 50µg/ml and 0.4µg/ml. The set-up were then kept in an incubator maintained at 37ºC for 48 hours before harvesting and parasitaemia determined microscopically from thin Giemsa-stained slides. IC_{50} values for the crude plant extracts were graphically determined from dose-response curves. Data was analyzed by ANOVA, student t-test and correlation analysis using Graph pad prism version 5.0. All $p < 0.05$ values were considered significant. All the three plants were shown to have antimalarial activity with *Rubia cordifolia* L. Hexane extract being the most active (IC_{50} =0.5517 µg/ml) against chloroquine resistant *Plasmodium falciparum*, W2. This same extract was effective against CQ-sensitive strain with IC_{50} value of 2.747µg/ml. Qualitative phytochemistry on the extracts revealed bioactive compounds being present including Alkaloids, Terpenoids and Flavonoids among others. These results indicate that the plant extracts possess antiplasmodial activity. It therefore confirms the antimalarial properties of the three medicinal plants. The study recommended isolation, identification and characterization of compounds and toxicity studies on extracts from the plants to act as lead molecules in the manufacture of effective antimalarial drugs.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Malaria is the commonest parasitic disease in humans. In 2016, nearly 3.2 billion persons were at risk of being infected with malaria and developing disease while a total of 216 million cases internationally occurred occasioning deaths of approximately 445 thousand people worldwide with 91% of the cases occurring in Africa (WHO, 2017). Majority of the affected were children below five years of age and largely in sub Saharan Africa (SSA) where malaria is believed to account for up to 50% of hospital admissions (WHO, 2005; Nyambati *et al*., 2013). It is estimated that 70% of the Kenyan population live in rural areas with 22 million people at risk of being infected with malaria and developing disease while 42 thousand persons among them 34 thousand children below five years of age die each year of malaria (DMS, 2006; Muthaura *et al*., 2015). As a result of inaccessibility and unaffordability of standard drugs, up to 80% percent of the people in African rural areas are largely dependent on herbal medicines (WHO2002b; cited by Muthaura *et al*., 2015). However, even with the extensive use of herbal treatments for managing malaria, little or no scientific data is available to justify the use of majority of them (WHO, 2002a; Muthaura *et al*.,2015)

As such there is need to evaluate traditionally used plants in order to validate their antimalarial properties. Furthermore, although chemotherapy of malaria is one of the key pillars in the control of the disease, both *Plasmodium falciparum and Plasmodium vivax* which are the deadliest causal organisms for human malaria have developed increased resistance to ordinary malaria medications and the resistance is spreading fast (WHO, 2007). This has in return rendered most of the previously first

line medicines ineffective, including Chloroquine, necessitating continued search for new medicines.

1.2 Problem Statement

Plasmodium falciparum, the most widespread causal agent of malaria in man is rapidly developing resistance to conventional anti-malarial medicines. Vaccination could offer a lasting malaria control solution but development of malaria vaccine is largely at preclinical phase currently (Greenwood *et al*., 2011). Furthermore, although chemicals are the cornerstone in malaria vector control, development of insecticide resistance has been rampant, rendering the usage of Long Lasting Insecticidal bed Nets ($LLIN_S$) and in-door residual spraying (IRS) non-viable mosquito control approaches (WHO, 2011). Declining efficacy of antimalarial drugs due to increase of resistant *Plasmodium* strains and the difficulty in developing malaria vaccine has created a need for different and novel anti-malarial remedies (Bloland, 2001; Nyambati *et al.,* 2013). The current study was therefore designed to investigate the activity of *Rubia cordiofolia* L., *Leucas calostachys* Oliv. and *Harrisonia abyssinica* Oliv. against *Plasmodium falciparum* 3D7 and W2 strains in order to validate their use as anti-malarial agents.

1.3 Justification of the study

Due to widespread drug resistance, new strategies are required to combat *P. falciparum* infections. Malaria is prevalent among the world's poorest population, who treat themselves with traditional herbal drugs. Herbal remedies are cheap and accessible to the majority of the malaria infected population (Nyambati *et al.,* 2013; 2015). Due to cultural believes these remedies are many a times believed to be more effective as compared to the conventional ones (Nyambati *et al*., 2013; 2015).

Although an *in vitro* study on anti-plasmodial activity of traditional medicinal plants in Kenya showed that extracts from *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica* Oliv. have anti-plasmodial activity against *Plasmodium knowlesi* (Nyambati *et al.,* 2013; 2015), no information is available on their efficacy against *Plasmodium falciparum*. This study evaluated antiplasmodial activity of aqueous, methanol and hexane extracts from the three plants namely *R. cordifolia* L., *H. abyssinica* Oliv. and L. *calostachys* Oliv. against *P. falciparum* 3D7 and W2 strains. *Plasmodium falciparum* was chosen because it the major cause of malaria and accounts for 99% of malaria cases (WHO, 2017). These plants were selected based on documented ethnobotanical information indicating that they are commonly applied for the management of human malaria among members of the Maasai community (Nyambati *et al.,* 2013). The plants are naturally abundant in the Maasai land natural forests particularly those of Transmara West sub-county and would therefore provide an alternative for the production of a safe, effective and affordable drugs against malaria. *In vitro* assays were preferred in this study because of the availability of protocol for *in vitro* culturing of *P. falciparum* (Trager and Jensen, 1976) and are used to determine the right dosage by directly assessing the drug performance besides being efficient, precise, and can allow evaluation of several compounds all at once (Kalra *et al*., 2006)

1.4 Research Questions

i. What are the anti-plasmodial effects of *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica* Oliv. extracts on Chloroquine resistant (W2) *P. falciparum* strain?

ii. What are the levels of anti-plasmodial efficacy of the extracts from *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica* Oliv. against Chloroquine sensitive (3D7) *P. falciparum* strain?

iii. What phytochemical compounds are present in *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica* Oliv. extracts?

1.5 Null Hypotheses

i. Extracts of *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica* Oliv. have no antiplasmodial effects on *P. falciparum* 3D7 (Chloroquine sensitive) and *P. falciparum* W2 (Chloroquine resistant) strains.

ii. There are no bioactive phytochemical compounds present in extracts of *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica*, Oliv.

1.6 Objectives

1.6.1 General objective

To investigate the activity of solvent extracts from *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica*, Oliv. against *P. falciparum* 3D7 (Chloroquine sensitive) and *P. falciparum* W2 (Chloroquine resistant) strains and identify phytochemicals present in the extracts.

1.6.2 Specific Objectives

i. To determine *in vitro* anti plasmodial effects of extracts from *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica* Oliv. on the growth of chloroquine resistant (W2) *P. falciparum* strain.

ii. To determine *in vitro* level of efficacy of extracts from *R. cordifolia* L., *L. calostachys* Oliv. and *H. abyssinica* Oliv. against the growth of *P. falciparum* 3D7 (Chloroquine sensitive) strain and compare it with that against *P. falciparum* W2 (Chloroquine resistant) strain.

iii. To qualitatively determine phytochemicals present in extracts from the three medicinal plants above.

1.7 Significance of the study

The study established that extracts from the three medicinal plants namely; *H. abyssinica*, Oliv. *L. calostachys* Oliv. and *R. cordifolia* L. possess antiplasmodial activity thus validating their herbal use. The study also demonstrated the presence of phytochemicals in the three plants, to which antiplasmodial activity is attributable.

CHAPTER TWO: LITERATURE REVIEW

2.1 Epidemiology of Malaria

Malaria has from time immemorial been among the most prevalent diseases affecting people particularly in the tropics where majority of the affected are young children aged below five years and pregnant women (Omole, 2011). In 2016, 3.2 billion persons were reportedly at risk of malaria worldwide with 216 million clinical cases and 445,000 deaths occurring (WHO, 2017). Majority of malaria cases occur in the sub Saharan (SSA) African countries where up to 90% of the deaths are attributed to malaria (WHO, 2014).

Malaria is most prevalent in tropical regions (Figure 2.1) around the equator due to the prevailing high humidity, high temperature, high rainfall and the resulting pools of stagnant water which provide ready breeding surfaces for malaria vectors (Jansen and Beebee, 2010). In eastern Africa, 25.3 million cases of malaria are reported annually where 8.2 million cases are from Kenya compared to 8.6, 5.3, 2.0, and 1.2 million cases for Tanzania, Uganda, Burundi and Rwanda respectively (Omole *et al*., 2011).

Despite the efforts to combat malaria in Kenya, it is still the leading cause of illness and death with 74% of the total population facing the risk of infection (Kenya Ministry of Health, 2014). The high infection rate could be attributed to insufficient medical care, emergence of drug resistant malaria parasites and insecticide resistance in malaria vectors (Masila, 2014). All the five *Plasmodium* species that infect man occur in Kenya namely *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax, Plasmodium malariae and Plasmodium knowlesi* with *P.*

falciparum being the deadliest of all to which up to 98% of malaria cases in Kenya are attributable (Kenya Ministry of Health, 2014).

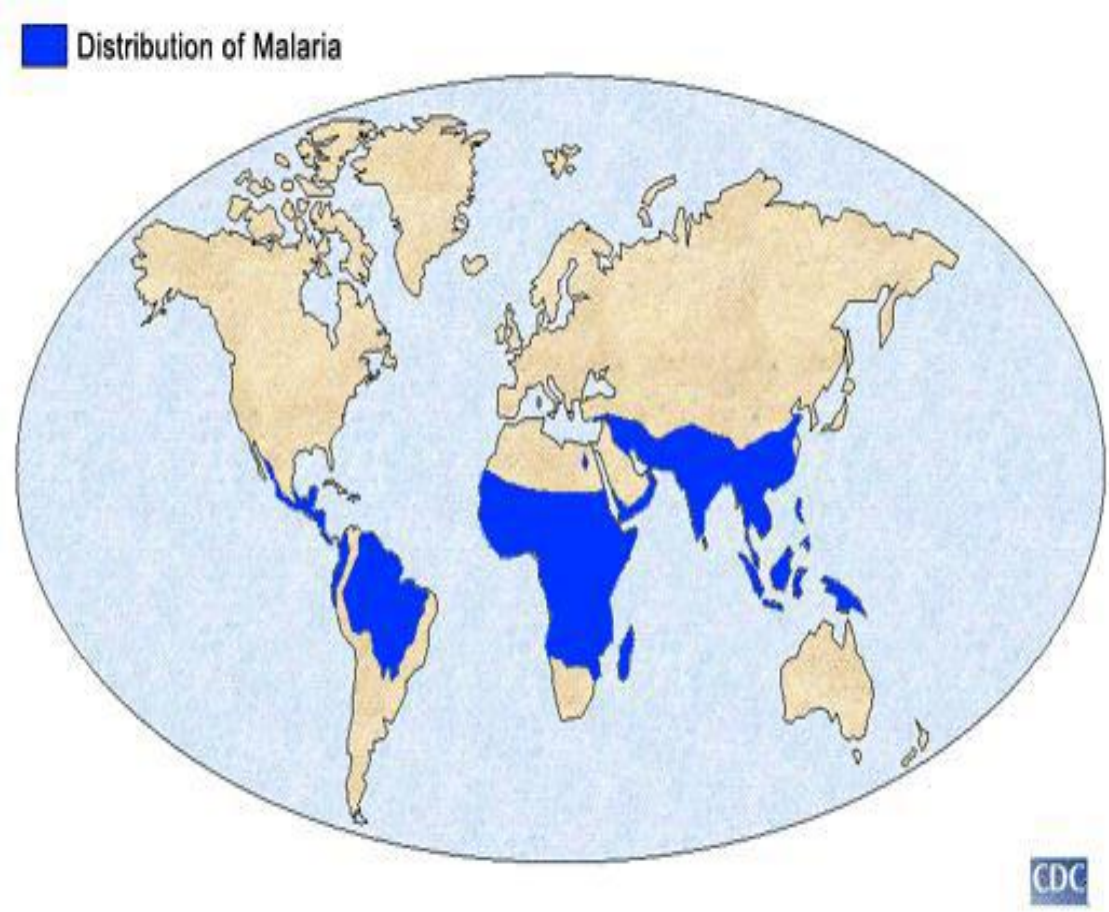

Figure 2.1: Map of world malaria distribution
(***http://www.cdc.gov/malaria/distribution epi/distribution.htm*, 2017**)

The transmission cycles of malaria are both perennial and seasonal and are determined by altitude, average temperature and patterns of rain. Low altitude areas (<1300M above sea level) are characterized by stable malaria transmission all the year round. These include coastal regions (Kilifi, Kwale, and Mombasa) and Lake Victoria basin (Kenya Ministry of Health, 2010). Medium altitude areas (1300M-1700M above sea level) including Kisii, Kuria and Transmara have unstable and seasonal transmission of malaria for 3-6 months annually due to seasonal rainfall. Areas of altitude ranging from 1700- 2500M above sea level experience malarial

epidemics and sporadic transmission cycles of 1-3 months per year. Areas above 2500M above sea level are malaria free zones because of low temperature that prevent vector breeding (WHO, 2016).

2.2 Economic cost of malaria

Malaria is prevalent and is associated with high mortality and morbidity translating into high costs of both treatment and control for affected individual and at government levels (Sachs and Malaney, 2002). The high morbidity and mortality in some countries with higher transmission rates has reduced per capita income by up to 1.3% and a 10% due to malaria burden. The cost of treatment and prevention is even higher as many productive man-hours are lost each day from those sick of malaria or taking care of such patients. Malaria infections in most pregnant women results into infant mortalities, miscarriages, low birth weight babies as well as increased chances of death due to suppressed immunity. Furthermore, the economic costs involved due to deaths from malaria are high, a part from the pain and suffering associated with the disease. In the long end, malaria negatively affects trade and foreign investors including tourists visiting regions with high infection risk (Omole, 2011).

2.3 General life cycle of *Plasmodium* species

Plasmodium species have a complex life cycle that occurs in both a vertebrate host, (human or animal) and an insect vector. The parasite enters the vertebrate host bloodstream when an infected female *Anopheles* mosquito bites (WHO, 1998). The cyclic development of plasmodium parasites proceeds through three distinct cycles (Figure 2.2): sporogonic cycle (occurs in the vector), pre-erythrocytic phase (takes

place in the liver cells of the vertebrate host) and erythrocytic cycle that occur in the red blood cells of the host.

2.3.1 The vertebrate phase

The *Plasmodium* parasites occur in the vertebrate host in both pre-erythrocytic, also called exo-erythrocytic stage and erythrocytic stages. Pre-erythrocytic cycle starts when an infected mosquito bites and hence inject sporozoites into a vertebrate host as it sucks blood. The sporozoites migrate to the liver within one hour and each enters a liver cell. Once within a liver cell, sporozoites transform into feeding trophozoites and mature within one week to start schizogony.

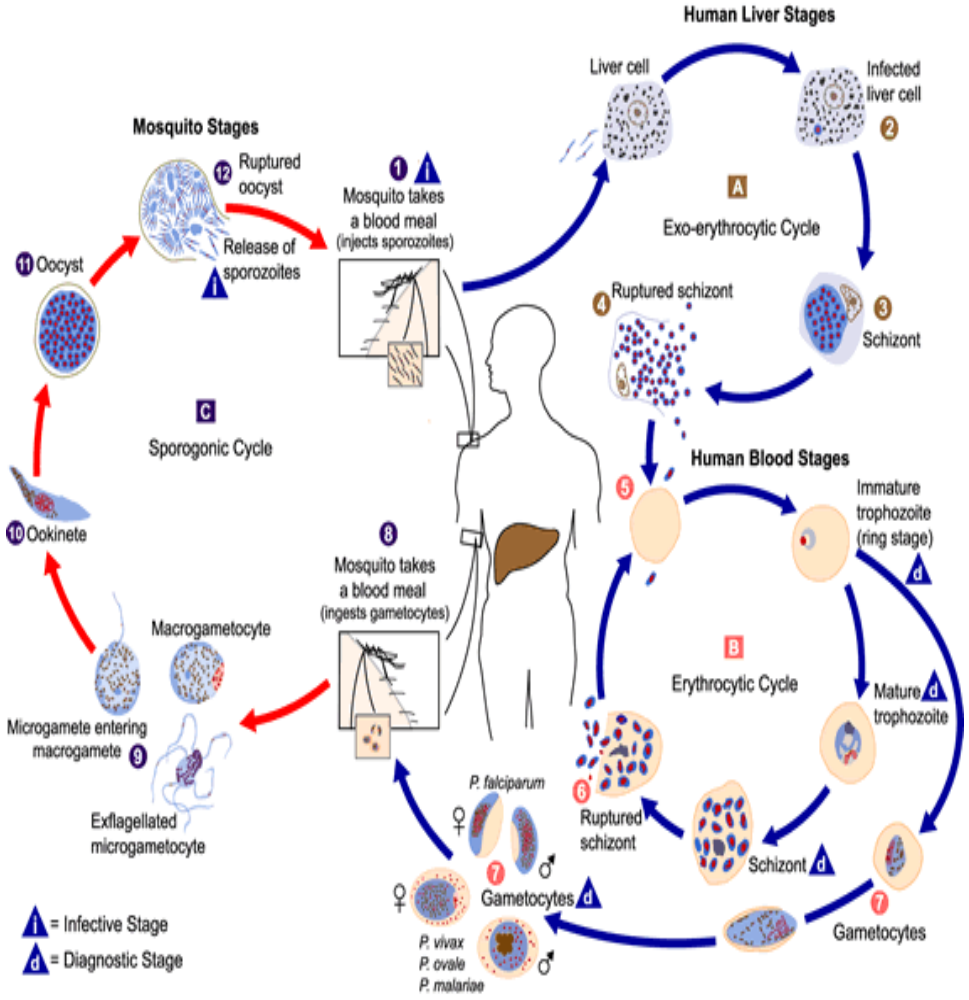

Figure 2.2: Generalized lifecycle of malaria parasites (www.cdc.gov/dpdx, 2015)

During schizogony, the trophozoites transform into tissue schizonts which later burst and release, a number of merozoites into the blood system of the host. This initiates erythrocytic phase. The merozoite in the red blood cells transform into trophozoites by ingesting haemoglobin and cytoplasm of the host. The parasite grows and fill more than half of the erythrocyte and through schizogony develops into mature schizonts, each consisting of an average of ten merozoites (Ozwara *et al.,* 2003). When merozoites are wholly developed, the red blood cells of the host burst hence releasing the merozoites and haemozoin. Many merozoites are destroyed by the immune system of the host while others re-invade uninfected red cells immediately (Larry and Gerald, 1996; Were *et al.*, 2010). Erythrocytic schizogony lasts for 1-4 days depending on the species. Schizogony lasts for 48 hours in *P. falciparum*, 25 hours in *P. berghei* and 24 hours in *P. knowlesi* (Garnham, 1996; Nyambati *et al.,* 2013; 2015).

After several asexual cycles, a small number of the merozoites change into macrogametocytes and microgametocytes which then appear in the peripheral blood system of the host within 8-11 days (Garnham, 1996). The sexual forms do not multiply but increase in size, almost filling the blood cell, and circulate in the body of the host until it is ingested by the next *Anopheles* mosquito that feeds on the blood from an infected host.

2.3.2 Invertebrate phase

The sexual cycle of the *Plasmodium* parasite take place inside the gut of the female anopheles mosquito. Once swallowed by the mosquito, gametocytes grow and distinguish into male and female gametes. However some of them are digested along with the blood or are phagocytized by the reticuloenothelial system of the

invertebrate host (Were *et al.*, 2010). The macrogametocytes then transform into macrogametes in a process that involves a shift of the nucleus towards the periphery. The micro gametocyte undergoes exflagellation. The microgametocyte then divides repeatedly forming six to eight daughters within a time span of 10-20 minutes to form the nuclei that develop into microgametes. The microgamete keeps swimming about until it finds a macrogamete with which it fuses and forms a zygote which then changes into ookinete that infiltrates the peritrophic membrane of the mosquito gut. It then rounds up below the epithelium as an oocyst for two to three weeks (Ozwara *et al.*, 2003). Meiosis occurs within the oocyst resulting in several haploid nucleated masses called the sporoblasts. The sporoblast yields numerous sporozoites by undergoing sporogeny. The oocyst bursts and the sporozoites drift into salivary glands. The sporozoites are injected into another host during the next blood meal. Inoculation into the new host marks the beginning of another cycle again. Development of sporozoites takes place between ten to fourteen days depending on the species and temperature. Once infected, a mosquito remains infective for life and retains capability to transmit malaria to every susceptible vertebrate it bites (Larry and Gerald, 1996).

2.4 The life cycle of *P. falciparum*

Plasmodium falciparum remains the most fatal among the five *Plasmodium* species causing malaria in man. As it sucks blood, an infested female anopheles mosquito injects between 5-20 *P. falciparum* sporozoites into the host (Rosenberg *et al*., 1990). The spororozoites migrate and arrive in the liver cells after a short while. Sporozoites then travel across several liver cells and then enter one; this marks the beginning of the liver stage of the parasite (Mota *et al*., 2002). The parasite matures within the liver in an average of 6.5 days giving rise to schizonts. The schizonts

stage then bursts and releases up to 30,000 merozoites for each sporozoite into the bloodstream. The merozoites are then transported all over the body of the host. Every merozoite that is not engulfed by phagocytic cells enters a host red blood cell beginning a cycle of replication that lasts for 48 hours. Schizont bursting and re-invasion of new red blood cells follows the replication process and this makes up the blood stage of *Plasmodium falciparum* (WHO, 2016).

Some of the merozoites undergo differentiation to form gametocytes. The gametocytes are swallowed by the female anopheles mosquito as it takes a blood meal. In the mosquito, the gametocytes undergo sexual reproduction to form zygote which turns into ookinete. Ookinete forms oocysts from which sporozoites are formed. The sporozoites then travel into the salivary glands and become infective (WHO, 2016).

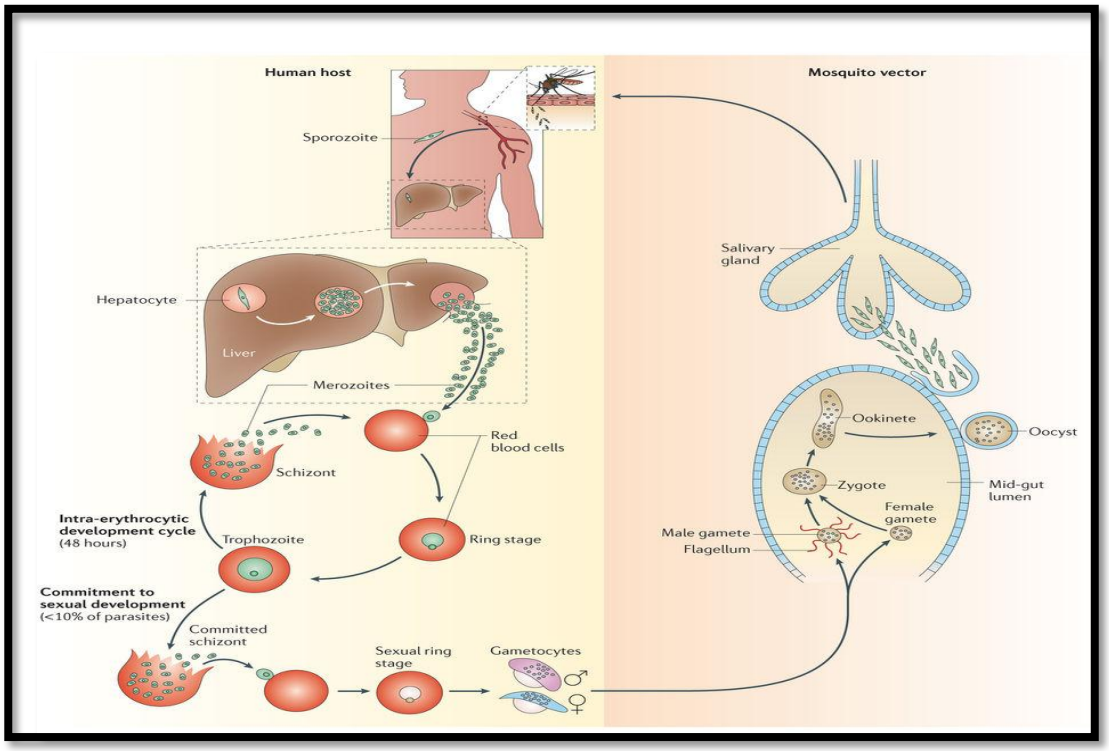

Figure 2.3: Life cycle of *Plasmodium falciparum* (*www.nature.com/nrmicro/journal v13/n9/figtab/nrmicro3519F1.html* , 2018

2.5 Control of malaria

Vector control through Indoor Residual Spraying (IRS) and sleeping under Long-Lasting Insecticide treated bed Nets (LLINS), development of new diagnostic procedures and effective chemotherapy employed in treating sick individuals, are the major malaria control strategies used (WHO, 2010)**.** Vector control targets reduction of Anopheline mosquito population at the larval and adult stage (WHO, 2013).

2.5.1 Indoor residual spraying (IRS)

Indoor Residual Spraying involves application of residual insecticides onto possible resting surfaces for malaria vector, the female anopheles mosquito (WHO, 2013). It targets reduction of adult anopheline mosquito population within the human habitation by using residual insecticides on the indoor surfaces so as to reduce human-vector contact. Malaria transmitting anopheline mosquitoes are endophagic and rest in the house after feeding during which their bodies absorb lethal concentrations of the insecticides, thus making IRS effective. However, it has been observed that IRS offers maximum community protection if above 80% of the houses in an area are covered and IRS operations reportedly protected 30 million people in 2012 in the 15 original presidents malaria initiative (PMI) countries (WHO, 2013). Natural pyrethrins from the flowers of *Chrysanthemum cinerariaefolium* have been used as powerful insecticides because they have low mammalian toxicity. Effectiveness of natural pyrethrins is dependent on the rapid knock down effect but lack of prolonged residual action has restricted their use (Kumar, 1984).

Due to widespread resistance to synthetic pyrethrins by mosquitoes, other synthetic insecticides including organochlorines such as Dichloro diphenyl trichloroethane

(DDT), organophosphates and carbamates have been used for mosquito control (Rozendaal, 1997). However, most Organophosphates are highly toxic to non-target species including man. In addition, widespread insecticide resistance in the competent malaria vector, *Anopheles gambiae* posses major threat to effective malaria control (WHO, 2011).

2.5.2 Use of long-lasting insecticide treated bed nets (LLIN)

The use of long lasting insecticide treated bed nets has proofed effective in reducing human host-vector contact with significant reduction of malaria transmission. The use of ITNS was reported to have reduced number of deaths due to malaria by over 44%, and also lowered the hospitalization of children due to malaria by 41% and reduced childhood deaths by 33% in Kenya. It was also estimated that the extensive dissemination and usage of ITNs could protect at least 500,000 children annually in Africa only. However, use of LLINs is suffering major setbacks ranging from widespread insecticide resistance in mosquitoes, failure of populations to use the nets, lack of the nets due to prohibitive costs and loss of physical integrity of the nets (WHO, 2003). As such there is need for more alternatives for malaria control and treatment.

2.5.3 Mosquito larval control

Larval source management is used to manage and reduce population of mosquito larvae before they emerge into disease transmitting adults. It involves using larvicides, Insect Growth Regulators (IGR), Biological Control Agents (BCAs) and Environmental Management (EM) (WHO, 1997). According to WHO (2014), EM for vector control involves planning, organization and handling of environmental factors or their relations with an aim of preventing or reducing vector development and decreasing contact of human beings with vectors. Environmental management is

achieved through building dams at high altitudes or far away from settlements, draining water in permanent (swamps) and temporary breading habitats (tyres, tins, water storage tanks), intermittent irrigation, desiccation by planting trees and improved housing (Ghebreyesus *et al*., 1999). However, environmental control has suffered a major setback since some vectors have undergone adaptations that make their larvae survive in temporary water collections in the holes of tree trunks, animal hoof prints, old disused tins, tyres and marshy grounds (Githeko *et al*., 2000).

2.5.4 Malaria chemotherapy

Chemotherapy involves use of conventional drugs to kill or inhibit growth of malaria parasites in the vertebrate host. Tolerability, affordability, accessibility in endemic countries, safety and short course regimens are among important attributes that drugs for successful malaria chemotherapy should possess (WHO, 2010)**.** Three major groups of antimalarial drugs used for malaria chemotherapy are quinolines, antifolates and artemisinin and its derivatives. The drugs differ on the basis of parasite stage targeted, metabolic pathway, half-live and the extent of parasite clearance.

2.5.4.1 Aminoquinolines

These are antimalarial drugs with an amine group substituting for a carbon atom in their molecular structures. They include, chloroquine, quinine, amodiaquine, piperaquine, mefloquine, atovaquone, halofantrine and lumefantrine.

2.5.4.1.1 Quinine

Quinine is among the first antimalarial drugs that has been in use for long in the management of fevers. Quinine was first isolated and identified in 1820 before synthetic substitutes such as chloroquine (CQ) were manufactured and used for

malaria treatment (Butler *et al*., 2010). Quinine remained in use as the first line drug for malaria treatment until CQ was developed. It is currently used together with antibiotics for treating malaria due to drug resistant parasites. It is administered through intravenous infusion or parenterally for patients who cannot retain oral intake. Besides clearing the blood of parasitaemia it also has antipyretic activities. Quinine is known for short half-life of about 8–10 hours probably due to lack of widespread quinine resistance. However *in vivo* emergence of quinine resistance has been reported (Briolant *et al.*, 2011). Quinine also has side effects such as depression in heart action, nausea, blindness, headaches, and hypersensivity reactions. Other side effects include cardiac dysrhythmias, central nervous system (CNS) disturbances ahypoglycaemia (Were *et al.*, 2010).

2.5.4.1.2 Chloroquine

Chloroquine (Figure 2.4) is aminoquinoline compound that was first identified in the 1940s and used in treatment of malaria. It had a high efficacy, affordability, safety even in pregnancy and was therefore used as a gold standard in the treatment of malaria for a long time (Alkadi *et al.*, 2007).

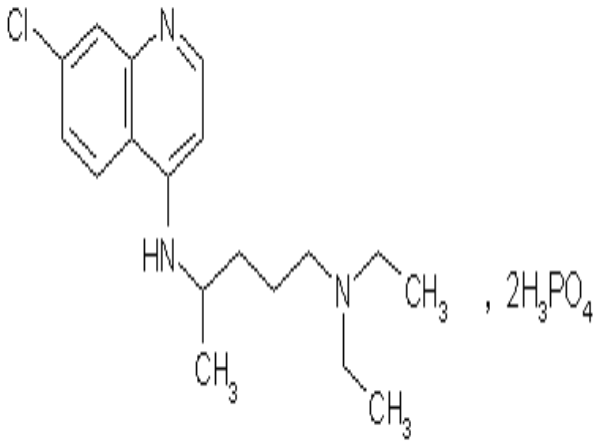

Figure 2.4: Structure of chloroquine (www.drugbank.ca/drugs, 2016)

Despite these positive attributes, Chloroquine has a long half-live, about 60 days. This may lead to selection for drug-resistance in the parasites due to their exposure over an extended time of activity long after the drug falls below its therapeutic concentration (White, 2008). However, chloroquine resistance was reported within 10 years after it had been introduced. Chloroquine resistance was first reported along Thai-Cambodian border and later in Papua New Guinea, and the Philipines (Mota, 2002). The resistance then spread fast and now it is in most areas known for malaria endemicity. However, chloroquine is still effective particularly in people who have developed partial immunity as a result of repeated infections and it is still retained for the treatment of malaria caused by *P. vivax*. Nevertheless, reports indicate that the dominance of chloroquine resistant *P. vivax* is increasing at a disturbing rate (WHO, 2010). Additionally, chloroquine has many side effects such as headache, nausea and vomiting, diarrhea, developing itchy rashes, pruritis, dizziness and blurring vision (Simooya *et al.,* 1998).

2.5.4.1.3 Amodiaquine

Amodiaquine is an aminoquinoline and its molecular structure is associated with that of chloroquine (Fig. 2.5). It has been in used for malaria control for over 70 years (White, 2008). Amodiaquine has a short half-life of about 3 hours (Petersen *et al.,* 2011).

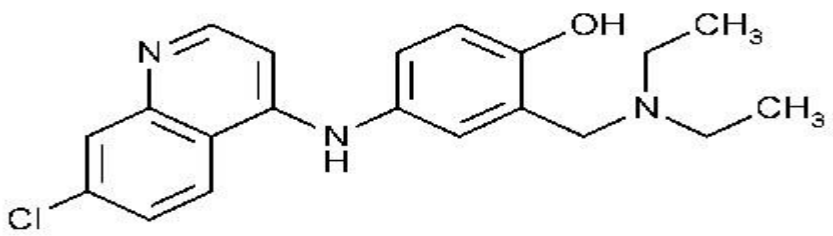

Figure 2.5: Structural formulae of Amodiaquine (*www. newdruginfo.com*, 2018)

Based on its structural similarity to chloroquine, cross-resistance mediated through mutations in *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) and *Plasmodium falciparum* multidrug resistance protein 1 (PfMDR1) has been reported. However, this cross-resistance is partial and some parasites exhibiting chloroquine resistance still remains vulnerable to amodiaquine (Ringwald *et al*., 2009).

2.5.4.1.4 Primaquine

Primaquine (Figure 2.6) is an aminoquinoline having a half-life of six hours that is recommended for the treating *P. vivax* hypnozoites (Wells *et al.,* 2010). Primaquine binds to PfCRT and stops the transport of chloroquine resulting into synergistic effect between itself and chloroquine hence helps in reversing the chloroquine resistance (Petersen, 2011).

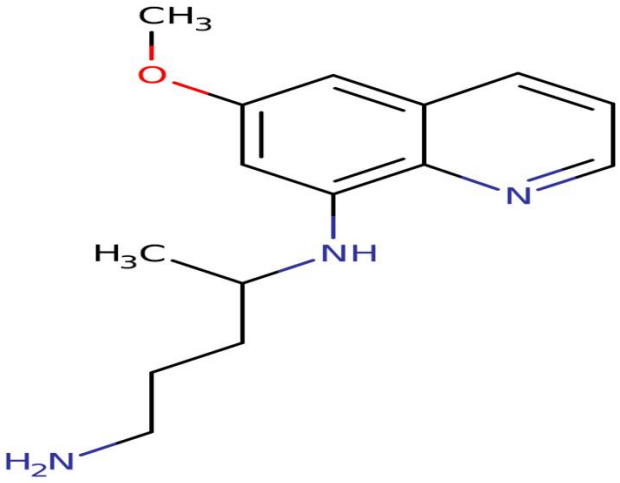

Figure 2.6: Structural formulae of Primaquine (*www.pubchem.ncbi.nlm.nih.gov*, 2018)

Primaquine is not recommended for people with deficiency of glucose-6-phosphate dehydrogenase since it is likely to cause haemolytic anaemia. Prevalence of glucose-6-phosphate dehydrogenase deficiency (G6PD) is varied from region to region but

ranges between 0.9–28.1% in Africa, about 2% in South America, 0.7–10.8% in Southeast Asia and 6.1–29% in the Middle East (Beutler *et al*., 2010).

2.5.4.1.5 Mefloquine

Mefloquine (Figure 2.7) is a methanolquinoline introduced in the 1970s characterized with a long half-life of about 18 days (White, 2008). Its mode of action involves binding onto the heme thus preventing detoxification which leads to death of the parasite (Eastman and Fidock, 2009). Mefloquine is a blood schizonticide affecting erythrocytic stages of malaria and hypnozoites when given in combination with Primaquine.

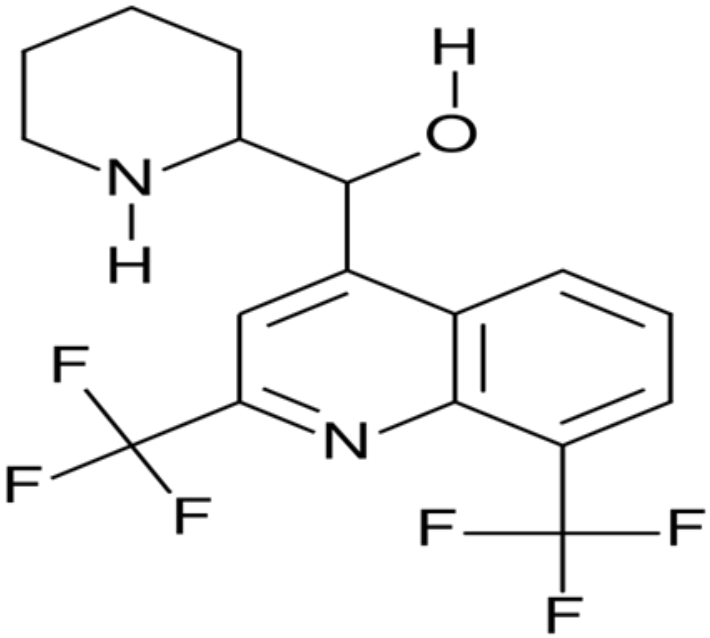

Figure 2.7: Structural formulae of Mefloquine (*www. newdruginfo.com*, 2018)

It is recommended for treating malaria caused by CQ-resistant *P. falciparum* and also for short-term chemoprophylaxis when entering CQ-resistance zones. However mefloquine has several side effects that include convulsions and insomnia, in addition to gastrointestinal disturbances (Were *et al*, 2011).

2.5.4.1.6 Piperaquine

Piperaquine (Figure. 2.8) is an aminoquinoline drug known to possess a long half-life of up to five weeks. It shares a lot of structural similarities with chloroquine, hence it is believed to share similar mode of action as that of chloroquine (Petersen *et al*., 2011). Piperaquine causes clumping of hemozoin in the trophozoites digestive vacuole (DV). It was intensively used in China in the late 1970s as a single therapy and this led to *P. falciparum* developing resistance against it. As a result, its use has since been discontinued.

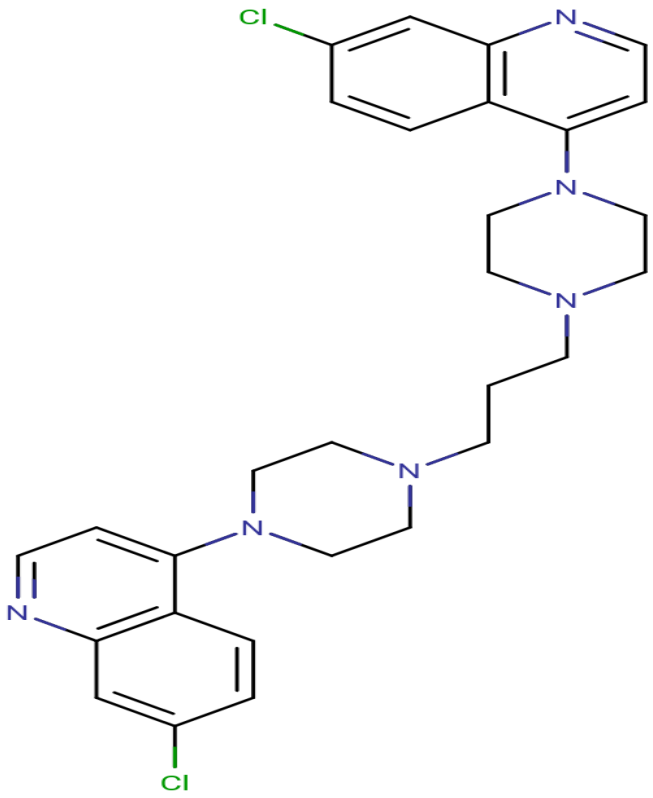

Figure 2.8: Chemical Structure of Piperaquine (*www. newdruginfo.com*, 2018)

2.5.4.1.7 Lumefantrine

Lumefantrine (Figure 2.9) is a blood schizonticide active against erythrocytic stages of *Plasmodium falciparum.* Lumefantrine inhibits the formation of hematin by

forming a complex with hemin and inhibits the formation of nucleic acid and manufacture protein (www.drugbank.ca/drugs, 2018).

It has a long a half-life of about 5 days and its uptake speed differs from one individual to another (Ezzet *et al.,* 1998). Lumefantrine requires to be taken together with a fatty meal in order to increase its oral bioavailability (Ashley *et al*., 2007)

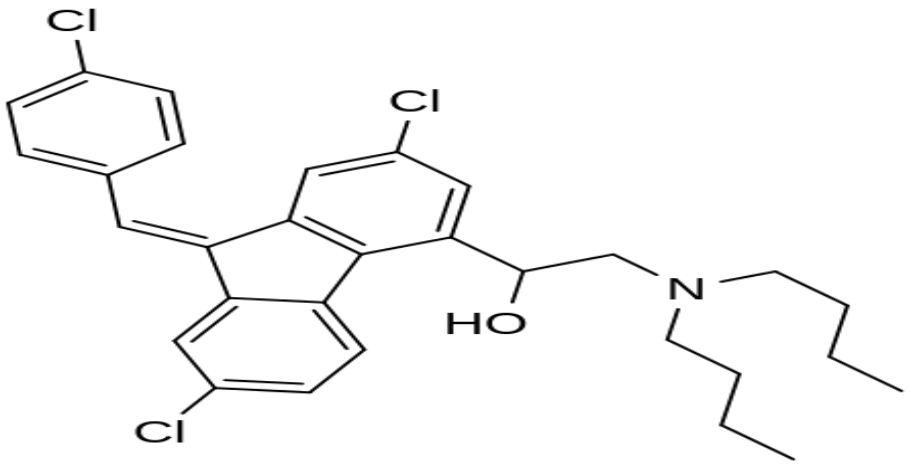

Figure 2.9: Lumefantrine formula (www.drugbank.ca/drugs, 2018)

2.5.4.1.8 Halofantrine

Halofantrine (Figure 2.10) is a blood schizonticidal drug effective against the blood stages of *P. falciparum* with resistance to CQ. Although halofantrine has good efficacy against *P. vivax* blood stages too, it has no activity against the hypnozoites. When taken through oral route, it is slowly and irregularly absorbed and attains the highest plasma concentration in about 5 hours. It is not used for malaria prophylaxis because it poses a risk of toxicity and it is unreliable in absorption (www.drugfuture.com). It is characterized with side effects which includes headache, abdominal pain, hemolytic anemia, convulsions, gastrointestinal

disturbances, sudden cardiac arrests and deaths and also transient rise in hepatic enzymes and convulsions (Nosten *et al.*, 200)

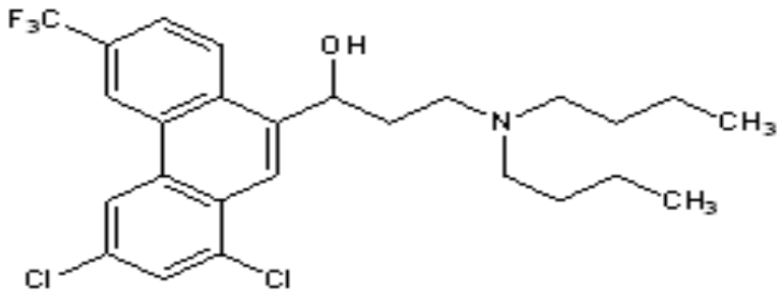

Figure 2.10: Structure of Halofantrine (*www.drugfuture.com*, 2018)

2.5.4.2 Antifolate drugs

These are drugs that inhibit the folate biosynthesis. Folate serves as a co-factor for one-carbon substitution reactions such as synthesis of thymidylate. Antifolates have similarity in structure to trimethoprim so can block its activity. Blockage of folate synthesis leads to halt of deoxyribonucleic acid (DNA) replication, reduced methionine synthesis and lower rate of converting glycine to serine due to decreased synthesis of pyrimidines.

The drug targets dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) that are both important in this pathway (Plowe, 2003). Proguanil may also target other pathways besides *Plasmodium falciparum* Histidine Rich Protein (PfHRP) (Srivastava and Vaidya, 1999). Pyremethamine have long half-life of 95 hours while sulphonamides have a half-life of 180 hours (Petersen *et al*., 2011).

2.5.4.3 Artemisinins

Artemisinin (AR) is an alkaloid, obtained from the *Artemisia annua* plant, the Chinese wood with the shortest half-life of 0.5-2 hours (Bloland and Barat, 1997). Artemisinin and its semisynthetic by-products have been in use worldwide as

alternative antimalarials since the quinolines and anti-foliates resistant strains of malaria parasites arose. Artemisinins possess an endoperoxide bond in its structure that gives it its antimalarial properties (Eastman and Fidock, 2009). Artemether (ATM), Artesunate (AS) and Dihydroartemisinin are the semi-synthetic forms of artemisinin (AR) and are most commonly used for clinical purposes owing to the low solubility of artemisinin (Bloland, 1997). Initial extensive use of derivatives of artemisinins as a single therapy in Southeast Asia led to the decline in efficacy that is currently being observed along the Thai–Cambodian boundary. The decline in efficacy being evidenced by the delay in the parasite clearance period is rapidly spreading to other malaria endemic areas (Eastman and Fidock, 2009)

2.5.4.4 Combined drug therapy

The rapidly emerging drug resistant *P. falciparum* presents an urgent need for alternative ways for the management of malaria infections. One such alternative is combination drug therapy. It is currently preferred in the treatment of malaria because it is likely to lower the rate of development and spread of resistance besides expanding the useful lifetime of the available drugs (WHO, 2001). Drugs combination is based on the fact that various drugs target different metabolic pathways such as amodiaquine and artesunate (AQ+AS). Some drugs target different stages of the parasite development such as amodiaquine (AQ) (blood schizonts) and Primaquine (gametocytes); also various drugs have different half-lifes with some having short half-life with rapid parasitaemia clearance, such as artesunate (AS) and Mefloquine (WHO, 2001). Combined therapy is advantageous because it is rare to have an individual infected with a parasite strain having resistance to two malaria drugs at the same time (White *et al*., 1998). Also, AR or its derivatives, having short half-life reduces the number of parasites rapidly hence

greatly reduces the chances of the parasites developing resistance to the other drug with which it is combined (Nosten *et al.*, 2000).

Artemisinin combination therapies (ACT_S) which combines semi synthetic forms of artemisinin are therefore currently, the best treatments for malaria. However the cost of artemisinin and its synthetic forms is high hence unaffordable to majority of the populations at risk. As a result of declining efficacy of conventional antimalarial drugs and rapidly spreading resistance by *P. falciparum*, there is an urgent need for new, affordable and more effective drugs preferably with novel mode of action.

2.5.5 Chemoprophylaxis

Some antimalarial drugs are used to prevent malaria especially among travelers to malaria endemic areas. The choice of a chemoprophylactic drug depends on the *Plasmodium* species and drug resistance prevalent in a country of travel (WHO, 2005). Primary prophylactic drugs are commended to start 2-3 weeks prior to departure and then continued during the entire period of stay in a malarias area and for further 1-4 weeks after arrival back home. Among the prophylactic drugs used are primaquine and proguanil which have been effectively used to stop the establishing of malaria infection in the liver by preventing pre-erythrocytic (PE) schizogony. Blood schizonticides on the other hand are used to suppress the blood forms of the malaria parasite. It is worth noting that none of the chemoprophylaxis is 100% effective yet and some may have slight side effects that hinder compliance (WHO, 2005; Were *et al.*, 2010).

2.6 The practice of Herbal Medicine

Herbal medicines, also called Complementary and Alternative Medicine (CAM) constitute traditional remedies that employ the use of therapeutic plant preparations

for treatment. The practice dates back to ancient times with Egyptians having used herbal medicine since around 1500BC. This indicate that herbal medicines is a derivative of both customs of ancient cultures and scientific knowledge (Kamboj, 2000).

Herbal medicines are assumed to be safe since knowledge about them has been accumulated over centuries. Furthermore, their chemical components being part of the biological functions of living plant life are compatible with the human body (Jeruto *et al*., 2015). Herbal medicines have gained favor in most regions of the developing countries where it has been shown to help people meet much of their primary health care needs. In China, 30%-50% of medicines are herbal medicines while in Nigeria, Ghana, and Mali herbal remedies form the first line medication for treating up to 60% fever cases in children (WHO, 2005).

2.7 Plants that have been studied for pharmacological activity

Plants are a rich source of medicines and some medicines currently used for treating malaria originated right from plants or molecules obtained from plants and used for template in their manufacture. Approximately 122 drugs from about 94 plants species were discovered via ethnobotanical studies (Fabricant and Farnsworth, 2001, Jeruto *et al.,* 2015). Most pharmacological medicines in use today comprise of a natural product model. Among the drugs is morphine, a pain-killer, derived out of *Papaver somniferum;* aspirin commonly used as analgesic drug was extracted from the willow plant bark while metformin used for the treatment of type 2 diabetes was extracted from *Melliotus officinalis.* Quinine, a malaria drug was isolated from *Cinchona ledgeria* while artemisinin was also obtained from *Artemisia annua* (Bailey *et al.*, 2007; Gathirwa *et al*., 2011; Jeruto *et al.,* 2015).

Similarly, the medicinal properties of crude extracts from many plants have been investigated by both *in vitro* and *in vivo* methods. In one such study the ethanol crude preparation of *Terminalia glaucescens* was found to have a higher inhibitory activity against chloroquine resistant strains of *Plasmodium falciparum* FcB1-Colombia, FcM29-Cameroon and a Nigerian chloroquine sensitive strains whose IC_{50} values were determined to be ranging between 0.35 and 0.55µg/ml (Mustofa *et al*., 2007).

Another study investigating the inhibitory effects of aqueous, methanol, butanol and ethyl acetate portions from *Terminalia avicennoides* extracts showed a high inhibitory effects for methanol fraction on *Plasmodium falciparum, 3D7 and K1* (both chloroquine sensitive) strains than the rest of the fractions (Shuaibu *et al.,* 2008). Kuria *et al*., (2001) also reported that ethanol macerate of *Ajuga remota* exhibited IC_{50} values of 55µg/ml against FCA/20GHA (CQ sensitive) and 57µg/ml against W2 (CQ resistant) strains of *Plasmodium falciparum* respectively. Additionally, methanol fraction of *Cleondendrum myricoides* also revealed a good *in vitro* antimalarial efficacy of IC_{50} = 10.2 µg/ml and 9.96µ/ml for D6 and W2 strains of *P. falciparum* respectively (Jeruto *et al*., 2015).

Following this trend, current study evaluated nine crude extracts from three different plants, namely *L. calostachys* Oliv., *R. cordifolia* L. *and H. abyssinica* Oliv. against *P. falciparum* 3D7 and W2 strains using *in vitro* antiplasmodial assays. These herbs are normally used by the Maasai people of Kenya to treat various forms of fevers (Nyambati *et al.,* 2013) hence the need to scientifically validate their antimalarial efficacy.

2.7.1 Ethnobotanical information on genus *Harrisonia*

The genus *Harrisonia* belongs to the family Simaroubacea. The family has approximately 30 genera and 150 species of shrubs and trees occurring in the tropics (Dutra *et al*., 1992). The stem bark and leaves of this family are characterized by bitter taste. The genus *Harrisonia* is comprised of three species namely; *Harrisonia abbysinica*, *Harrisonia perforata,* and *Harrison brownii*. *Harrisonia perforata* occurs in North Australia and China (Chen *et al.*, 1997) while *H. brownii* is found in the southern and south eastern Asia and middle Vietnam (Ridle *et al*., 1976). The plants in the family Simaroubacea have been used widely for treating viral infections, tuberculosis and malaria (Masila, 2014).

Harrisonia abyssinica grows in tropical Africa to a height of 6M. It is an evergreen plant with branches bearing curved or straight spines that usually occur in pairs. Its flowers are cream or yellow in color while the fruits are red when ripe (Appendix I). The species is threatened by overexploitation for medicinal purposes (Balde *et al*., 1995). Hot leaves decoction from *Harrisonia abyssinica* is prescribed for treatment of headache among the Nyamwezi people in Tanzania (Schmelzer *et al*., 2010). The Boro people of Ethiopia prescribe the root decoction for the treatment of dysmenorrhea (Schmelzer *et al*., 2010). In Kenya, *H. abyssinica* is the most widely used medicinal plant by herbalists in the Luo community living in western Kenya who use root barks decoction for treatment of fevers and sexually transmitted diseases (Kokwaro *et al*., 1993). Among the Maasai, Kuria and the Luo communities in Kenya, it is commonly used to treat malaria (Nyambati *et al.*, 2013). However, antimalarial efficacy of *H. abyssinica* Oliv. (Appendix I) from these regions against the most lethal malaria parasite, *P. falciparum* has not been documented.

2.7.2 Ethnobotanical information on *Leucas calostachys* Oliv.

Leucas calostachys Oliv. belong to the family Lamiaceae, and genus Lamiacae. *Leucas calostachys* Oliv. is a herb that is erect and with densely pubescent stems and leaves. It has opposite leaves that are shortly staked with about 4 cm length. Its flowers are white with longer corolla than the calyx (Appendix II) (Kokwaro, 1993). In Ethiopia *L. calostachys* leaves are commonly used in treating malaria, itchy skin and to stop bleeding. On the other hand the roots are used in treating amoebic dysentery and cough (Jansen and Cardon, 2005). In Kenya, it's crushed roots and leaves in water is drunk to treat stomachache among the Luo community in western Kenya (Kokwaro, 1993).

Leucas calostachys has also been indicated for the treatment of fevers and sexually transmitted diseases among the Luhyia people of Vihiga County in western Kenya. Here, a decoction from the leaves decoction is administered through drinking as a treatment for sexually transmitted infections (STIs) (Omondi *et al*., 2014). According to Jeruto *et al.* (2015), *L. calostachys* is used among Keiyo community for the treatment of heart burn and peptic ulcers. Its leaves are pressed and used to treat abdominal distension and heart problems while its leaves and roots are used in treating malaria. Among Sabaot community in Mt. Elgon area in Kenya, pounded leaves of *L. calostachys* Oliv. are used to treat headache and colds (Okello *et al*., 2010) which are among the symptoms of malaria.

In Transmara West District, largely occupied by the Maasai community, *L. calostachys* Oliv. is used to treat malaria. In this community a large proportion of members of this community (72%) use herbal medicine for disease management (Nyambati *et al.*, 2013; 2015). As such there is need to evaluate these medicinal

plants for antiplasmodial activity against *P. falciparum* in order to validate the herbalists' claim that it cures malaria.

2.7.3 *Rubia cordifolia* L.

Rubia cordifolia L. (Appendix III) is a perennial, prickly, climbing herb belonging to family Rubiaceae. Leaves are arranged in a whorl of four with quadrangular petioles which are sometimes also prickly on the angles. Leaves do not bear stipules while the stem is slender, rough, and with four angles and bearing sharp curved prickles on the ridges. Flowers occur in cymes and are green-white in colour. Fruits are didymous or globose, smooth, shining and purplish black when ripe (Priya and Siril 2014).

Rubia cordifolia has been indicated in blood purification and is extensively used against blood, skin and urinary tract infections (Sivarajan *et al.,* 1994). The root extract is used against several health problems including arthritis, cough, diabetes, skin discolouration, dysmenorrhoea, jaundice, tuberculosis, general body debility, intermittent fevers, leucorrhoea, splenopathy, slow healing broken bones and urethrorrhoea. However, the use of roots for these pharmacological purposes is not sustainable as it involves uprooting the plant which can lead to its death. Among the Maasai people of Kenya, it has been indicated for the treatment of Malaria (Nyambati *et al*., 2013)

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study site

The study was conducted at the Institute for Primate Research (IPR) located in the Oloolua forest in Nairobi, Kenya. The plant materials were harvested from the natural forest in Transmara West-sub county in Narok County, Rift Valley region, lying within $1^0$00´S and $34^0$52´E (Appendix IV). The plants were taxonomically identified at the East African Herbarium, National Museums of Kenya (NMK), Nairobi. Extraction was done in the pharmacognosy laboratory in Kenyatta University. The *in vitro* assays and qualitative phytochemical analysis were done in the IPR malaria Laboratory.

3.2 Study design

The study utilized randomized block experimental design involving *in vitro* culture bioassays. The *in vitro* assays to test the anti-plasmodial activity of each extract of these plants against W2 and 3D7 strains of *Plasmodium falciparum* were performed in triplicates in 12 x 8 flat bottomed well microtitre sterile plates (COSTAR®) (Appendix V). The study employed quantitative method of data collection to determine antiplasmodial activity and qualitative method to determine anti-plasmodial phytochemicals present.

3.3 Collection and preparation of plant materials

Leaves of *H. abyssinica* Oliv. (Appendix I), *L. calostchys* Oliv. (Appendix II) and *R. cordifolia* L. (Appendix III) were collected from various sites in Transmara West sub county (Appendix IV) largely occupied by the Maasai community in the month of April 2015. The harvested parts were dried under shade for 14 days and then ground into powder using a hammer mill (8 LAB MILL CHRISTY AND MORRIS LIMITED, ENGLAND) at Technical University of Kenya. The plant materials were

packed into clean air tight plastic containers, stored in a dark, cool and dry place before transporting them to the pharmacognosy laboratory at Kenyatta University for solvent extraction. The plants were selected based on documented ethnobotanical information (Nyambati *et al.,* 2013; 2015).

3.4 Solvent extraction

Solvent extraction was carried out at Kenyatta University, laboratory of pharmacognosy. Organic solvents n-hexane and methanol were purchased from Kobian Scientific Ltd, Nairobi, Kenya while distilled water used was obtained from the reproductive health Laboratory. All the organic solvents and reagents used for extraction and phytochemical analysis were of Analytical grade.

3.4.1 Hexane extraction

The sample extraction followed the procedure described by Harbone (1984). Briefly; one hundred and fifty grams (150g) of dry powdered plant material was weighed (Mettler PM 300) and transferred into a clean dry glass beaker. Two hundred and fifty (250) ml hexane was added to cover the material under the fume hood and was left to soak in the solvent for 48 hours with constant shaking at room temperature. The mixture was filtered under pressure using a vacuum pump and whatman filter paper number 1. The filtrate was rotor vaporized using rotor evaporator (BUCHI, SWITZERLAND) and dried to a constant dry weight. It was then transferred into a pre-weighed sample bottle which was stored in -20°C till required for bioassays (Muregi *et al*., 2014).

3.4.2 Methanolic extraction

Hundred and fifty grams (150g) of dried and powdered plant material was weighed and transferred into a clean and dry glass beaker. Two hundred and fifty (250) ml

methanol was added to cover the plant material under the fume hood and left to stand for 48 hours with constant shaking at room temperature. The mixture was filtered using vacuum pump and whatman filter paper No.1. The Filtrate was rotar vaporized (BUCHI, SWITZERLAND) to concentrate it and dried to a constant dry weight. It was then transferred into a pre weighed and air tight sample bottle and kept at -20°C till required for bioassays (Muregi *et al.*, 2004).

3.4.3 Plant water extraction (Aqueous extract)

One hundred and fifty grams (150g) of the plants dry sample was weighed. Distilled water was added to cover the plant material (250ml) and then kept in a water bath maintained at 60ºC for 24 hours. The mixture was filtered (whatman filter paper No.1) into a plastic bottle and the filtrate stored in a freezer at -20ºC to prevent contamination and also for cold preservation. The extract was then thawed by placing the bottle in a warm water bath at 70ºC and then transferred into a round-bottomed rotor evaporator flask. The flask was fixed to the freeze dryer at a vacuum pressure of -1 and a temperature of -40ºC for two days to give a concentrated extract in powder form. The extract was then weighed, its weight recorded and then kept at a temperature of -20ºC till needed for *in vitro* assays and phytochemical analysis (Muregi *et al*., 2004).

Table 3.1: Plant extracts prepared for bioassays with *P. falciparum*

Plant name	Extraction solvents used			Extractions
	Hot water	**Methanol**	**Hexane**	
R. cordifolia	1	1	1	3
L. calostachys	1	1	1	3
H. abbyssinica	1	1	1	3
Total extractions	3	3	3	9

1, 3= Number of extracts per solvent and plant respectively 9 = Total number of extracts tested.

Plant leaves were used in all cases so as to replicate the herbal practice among the Maasai in Transmara West Sub County.

3.5 The parasite

In this study 3D7 (CQ sensitive) and W2 (CQ resistant) strains of *P. falciparum* were used. The parasite strains were obtained from the Malaria Parasite Bank, IPR, in Nairobi, Kenya. Parasite cultivation for *P. falciparum* W2 and 3D7 strains was done following Trager and Jensen (1976) procedure.

3.5.1 Preparation of human serum

In preparing human serum, the method by Trager and Jensen (1976) was used. From the donor, venous blood was aseptically drawn into a bleeding bag free of anti-coagulant and left to clot at room temperature for 90 minutes before being transferred to 4ºC overnight (18 hours). The haemoserum was dispensed into 50 ml centrifuge tubes (CORNING®) and then centrifuged at a speed of 1500 revolutions per minute (rpm) for 10 minutes at room temperature. The haemoserum was aliquoted into separate 10ml sterile tubes (CORNING®) in which it was heat inactivated for one hour at 56ºC. The haemoserum was then stored at -20ºC and used within six months.

3.5.2 Processing of uninfected erythrocytes for culturing of the parasite

Uninfected erythrocytes were prepared according to Trager and Jensen (1976). Briefly, blood group O rhesus positive from recruited volunteer donor at the Kenya Institute of Medical Research (KEMRI) was drained into a sterile 50 ml centrifuge tube (CORNING®) containing 15%(v/v) Acid-citrate –dextrose (ACD) anticoagulant. It was determined that blood donated was malaria, HIV and hepatitis free before donation. It was also established that the donor had neither taken antimalarial drugs nor antibiotics in the previous two months. The blood was centrifuged (BECKMAN GPR CENTRIFUGE, USA) for 10 minutes at a speed of 1500 revolutions per minute (rpm) at room temperature. The plasma together with the white blood cells layers were aspirated using a sterile cotton wool plugged 10 ml pipette and discarded. This was done in a Class II biological safety hood at the IPR. The red cell pellet was then washed thrice in two times the original volume of RPMI 1640 (GIBCO). The resulting suspension was then centrifuged for 10 minutes at 1500 rpm before aspirating and disposing of the supernatant each time. The pellet size was determined using 1000µl (Gilson France) micropipette and then an equal volume of RPMI 1640 (GIBCO) added to obtain a 50% haematocrit before the mixture was gassed with 92% nitrogen N_2, 5% Carbon (IV) Oxide (CO_2) and 3% Oxygen (O_2) gas mixture (BOC, KENYA). The red blood cells so prepared were then stored at 4ºC for use within 28 days for *in vitro* culturing of *P. falciparum* W2 and 3D7 strains (Muregi *et al*., 2004).

3.5.3 Incomplete culture medium (ICM)

The method of preparing ICM was adopted from Rowe *et al*., (1968) whereby 90.4% v/v of RPMI 1640 was mixed with 3.8% HEPES, 3.8% of 5% w/v sodium bicarbonate solution and 1% v/v of 20% w/v D-glucose.

3.5.4 Complete culture medium (CCM)

The complete culture medium (Appendix V) was prepared by mixing together 90% (v/v) incomplete culture medium and 10% heat inactivated haemoserum stored at 4ºC for use within one week after preparation (Rowe *et al.,* 1968; Omole, 2011).

3.5.5 Thawing of the malaria parasite and setting of the culture.

The malaria parasites were prepared using Rowe *et al.,* (1968) methodology. Laboratory adopted *P. falciparum* (W2 and 3D7) parasites preserved in liquid nitrogen within the malaria unit at the (IPR) were retrieved and speedily warmed up in a water bath kept at 37ºC and then transferred into the Biosafety hood (Class II). The ampoules were surface sterilized by swabbing the surface with cotton gauze soaked in ethanol (70%). The stocks were then lightly disturbed and moved into a labeled 50ml centrifuge tube (CORNING®) using 1ml sterile pipette while still cold and 0.2 ml of filter sterilized 12% (w/v) sodium chloride solution added drop wise. The mixture was left undisturbed for 5 minutes. It was followed with 10ml of filter sterilized 1.6% sodium chloride solution in distilled deionized water added drop by drop while gently shaking. This was also subsequently followed by 10ml of 0.9% sodium chloride added while gently shaking. The cells were centrifuged for 10 minutes at a speed of 1500 rpm (BECKMAN GPR CENTRIFUGE, USA) at room temperature (24 ºC). The supernatant was aspirated using sterile 10ml pipette and discarded into 10% JIK. The packed cells were immediately re-suspended in 5ml RPMI, centrifuged followed by aspiration and discarding of the supernatant. Lastly, parasites were suspended in 5 ml Complete Culture Media before it was centrifuged, supernatant aspirated and discarded.

The packed cell volume (PCV) of the washed parasites was determined using 1000 µl micropipette (Gilson, France). Complete culture media (CCM) (Appendix V) was

added to adjust hematocrit (hct) to 2.5%. The cultures were gassed (BOC, Kenya LTD) before incubation at 37 ºC. The culture medium was changed after 48 hours intervals to refresh nutrients and also remove the toxic metabolic waste products. Smears were subsequently prepared and observed for parasitemia determination, growth rate estimation and to check for possible presence of bacteria or fungi.

3.5.6 Refreshing of *in vitro Plasmodium falciparum* cultures

Briefly, about 100 µl of the culture was aspirated out under sterile conditions, dispensed into a labeled eppendorf tube and subsequently spun in a micro centrifuge (Beckman, USA) at 3500 rpm for one minute. The supernatant was removed and thrown away before the erythrocytes pellet was used to make thin smears for observation under 100X objectives for parasitaemia determination. Subsequently, the culture was transferred to labeled and sterile 50ml centrifuge tubes followed by spinning for 10 minutes at 1500 rounds per minute at room temperature (24 ºC).

The used culture medium was sucked off using sterile 10ml pipette and replaced with fresh complete culture media. A fresh culture medium added consisted of 10% sterile heat inactivated haemoserum in RPMI 1640 (GIBCO) and 25 µl gentamycin (GIBCO) to prevent microbial contamination of the culture. Fresh erythrocytes were added when the parasitaemia was high (>2.5%). Once fresh medium had been added, the culture was gassed with culture gas mixture containing 3% O_2, 5% CO_2 and 92% N_2 (BOC, KENYA) after which the culture was transferred to an incubator maintained at 37 ºC.

3.5.7 Studying the Growth Characteristics of *Plasmodium falciparam* 3d7 And W2 Strains

Both 3D7 and W2 strains of *P. falciparum* were cultivated and used to determine the chemotherapeutic effects of plant extracts. Prior to their expossure to the extracts,

the parasite strains were maintained in a continous culture over a period of three months. The parasites were then monitored for parasitaemia growth every day for purposes of exposing them to the extracts for one week and attained a parasitaemia of about 10% between the fourth and seventh day post incubation. The parasites were exposed to the drugs and extracts when they attained exponential phase of growth. Any change in growth pattern thereafter could be attributed to the effect of the drugs and the extracts.

3.5.8 Cryopreservation of the *Plasmodium falciparum* parasites

This was done according to Schichtherle *et al*. (2000). Cryopreservation was carried out to guarantee sufficient quantity of laboratory adopted isolates and also to have manageable culture flasks. Briefly, thin smears were made for observation to establish that the culture to be preserved did not have any contamination and that it had a high parasitaemia of 5% mainly at ring stage. The culture to be cryopreserved was centrifuged at 1500 rpm for 10 minutes at 24 ºC in a 50 ml centrifuge tube and supernatant aspirated. Packed cell volume (PCV) was then estimated and haemoserum gently added drop by drop while mixing, followed by the freezing medium (Appendix V) added drop by drop to 2:3:5 (cell Pellet : Haemoserum : Freezing medium) ratio while gently shaking. The culture was divided into fractions of 0.25 ml then transferred into separate 2 ml sterile cryovials (Grienen bio-one). The cryovials were then placed in aluminum canes (Nalgene cryo 1ºC /Min and stored at -80 ºC overnight before they were moved into liquid nitrogen freezer.

3.6 Plant extracts preparation

The plant extracts were first removed from the freezer at -20 ºC before 10 mg of each were separately weighed (Mettler PM 300). The weighed extracts were then separately dissolved in RPMI 1640 (GIBCO) to make the 10ml of 1mg/ml stock

solution following Nyambati *et al.* (2013) procedure. To each plant extract, 50 µl of Dimethyl Sulfoxide (DMSO) was first added and then vortexed for one minute before the volume was adjusted to 10 ml with RPMI 1640 (GIBCO). Chloroquine (CQ) and Artemether (ATM) standard drugs were also prepared both at a concentration of 1mg/ml concentration using water and 70% ethanol as solvent for Chloroquine and Artemether respectfully. The stock solutions were filter sterilized using 0.45 µm and 0.22 µm pore size micro filters (Minisart® CE) and then stored at -20 ºC till required for assays.

3.7 Extracts *in vitro* antiplasmodial activity testing

The crude extracts and control drugs sensitivity testing for *P. falciparum* W2 and 3D7 strains was done using Desjardins' semi-automated micro dilution technique (Desjardins *et al*., 1979). Sterile 96-well microtitre plates (COSTER®) were used in this study (Appendix VI). The complete culture medium (CCM) was dispensed into row B of the microtitre plate wells down to row H exempting row A. Stock solution of each extract was diluted in RPMI 1640 to 50µg/ml. Fifty (50) µl of the dilute extracts was added to row A in triplicate using a 1-200 micropipette (Pipettor, Pipetman Gilson, France). Using a multichannel pipette (Thermoscientific Finnpippete® Finland), two-fold serial dilutions were done by moving 25 µl from row A down to row H and the last 25 µl from row H discarded. This resulted into wells in row A having highest concentration of 50µg/ ml, well B having 25 µg/ml and well C, 12.5 µg/ml with the concentrations halving down to row H. The concentration range of 50µg/ ml to 0.4 µg/ml as the lowest concentration was therefore used. A similar process was carried out for the reference drugs (ATM and CQ) with concentrations range of 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039, 0.002 and 0.001 µg /ml. Three columns were used for each extract

and positive control drug and one plate therefore accommodated 4 drugs. Eight extract free wells also were included to provide reference IC_{50} (Negative control).

3.8 Addition of the parasite strains into the wells.

The *P. falciparum* strains (W2 and 3D7) were added into the wells following Desjardins *et al*. (1979) procedure. The culture was used when it was mainly at ring stage with no gametocytes. The parasites were first examined under a microscope to determine the parasitaemia level of the cultures. As the percentage parasitaemia for both cultures was higher, addition of 50% RBC was done to adjust it to 1% and Complete Culture Media (CCM) added to adjust the haematocrit to 1.5%. 200 µl of the resulting diluted culture was added into all the wells such that the total volume per well was 225 µl. After placing the lids of microtitre plates gently, the plates were transferred into airtight chamber which had a dump paper towel to maintain humidity around the cells. The lid of the gas jar was placed back and chamber gassed with a culture gas mixture (92%N_2, 5%CO_2, and 3% O_2; BOC, Kenya LTD) for three minutes and then transferred into an incubator maintained at 37 ºC for 48 hours. The components of every well was then harvested into a labeled 1.5 ml eppendorf tube, centrifuged for 3 seconds and then the supernatant was aspirated out. The pellet was pipetted onto a microscope slide labeled with date, investigator's initials and contents of the well and used to make a thin smear. This was stained for 15 minutes in 10% Giemsa solution after which developed schizonts were counted against the total 2000 erythrocytes.

3.9 Determination of the Inhibitory Concentration (IC_{50})

The concentration of every herbal extract that inhibited 50% growth of parasite strains was calculated as a fraction of the starting parasitaemia in the treated wells relative to the growth in the negative control wells from the starting parasitaemia of

1.0%. Parasitaemia determination was done by examination of giemsa-stained thin smears. Parasitaemia suppression and growth inhibition was calculated as the difference between the mean percentage growth in the negative control wells and percentage growth of each test well using the formula:

$$AV\%P = \frac{AV\ Ctrl - AV\ Treated}{AV\ Ctrl} \times 100$$

Where AV % P is Average percentage suppression of parasitaemia, AV Ctrl is average parasitaemia in the control wells and AV treated is the average parasitaemia in treated wells (WHO, 2001; Ngemenya *et al*., 2006). Antiplasmodial activity of both the crude extracts and control drugs (CQ and ATM) was expressed in terms of 50% inhibitory concentration (IC_{50}) determined from dose response curves by nonlinear regression analysis using graph pad prism version 5.0. Antimalarial activity of the drugs was classified using the activity scale of good (IC_{50}<5 µg/ml), moderate (IC_{50}>5 µg/ml to<50 µg/ml) and mild (IC_{50}>50 µg/ml (Muthaura *et al.*, 2015).

3.10 Phytochemical Investigation of the plant extracts

The crude extracts of each solvent from all plants were analyzed for the presence of different phytochemical components as outlined below.

3.10.1 Test for acids

The presence of acids was analyzed according the procedure of Ruskin *et al*. (2014). Briefly, to 1ml 1mg/ml of extract in a test tube, 1ml of 1M sodium hydrogen carbonate was added drop by drop using a dropper. Evolution of a gas (effervescence) would be an indicator of the presence of acids.

3.10.2 Betacyanins

To 2ml of 1mg/ml of each plant solvent extract in a test tube, 1ml of 1M sodium hydroxide was added. The mixture was then heated at 100ºC for 5 minutes. Appearance of yellow coloration is an indicator of betacyanins present (Harbone, 1973).

3.10.3 Test for quinones

Quinones were tested according Evans (1997) procedure. To 1ml of 1 mg/ml of each plant extract, 1 ml of concentrated sulphuric acid was added and allowed to stand for 5 minutes. Appearance of red pigmentation was a suggestion of the presence of quinones in the extract.

3.10.4 Test for coumarins

Ammonia solution was added onto separate filter papers, three drops each. This was then separately followed with a drop of 1mg/ml of each extract. The filter paper was observed against light. Fluorescence specified the presence of coumarins (Ruskin *et al*., 2014).

3.10.5 Test for proteins

Biuret test (Ruskin *et al*, 2014) procedure was used. Briefly, 1ml of 1mg/ml of each extract were separately treated with 1ml of 10% of sodium hydroxide and the mixture heated gently. This was followed with one drop of 0.7% copper (II) sulphate. The presence of proteins was indicated by appearance of purple color.

3.10.6 Test for reducing sugars

Reducing sugars were tested using Benedict's test (Ruskin *et al.,* 2014). Two (2) mg of each plant extract was transferred into separate test tube followed with addition of 10 ml of distilled water. The mixture was filtered and the filtrate concentrated by evaporation. Five milliliters of Benedict's solution was added before the mixture

was heated for 5 minutes in a water bath. Formation of brick red coloured solid showed that reducing sugars were present.

3.10.7 Test for fixed oils and fats

This was done using Stain Test (Ruskin *et al*., 2014). One hundred milligrams of each extract were separately transferred onto a filter paper and pressed against another filter paper. Formation of translucent mark on the filter papers showed that oils and fats were present in the extract.

3.10.8 Testing for flavonoids

Ferric chloride test

Presence of flavonoids was tested using Ferric Chloride Test (Raman, 2006). Briefly, 2ml of each solvent extract at 1mg/ml was separately mixed with 1ml of Ferric chloride ($FeCl_3$) solution. Appearance of a black-red colour would confirme flavonoids to be present in the respective extracts.

Alkaline reagent test

The presence of flavonoids in the crude extracts was also tested following Alhadi *et al*. (2015) procedure. Briefly, 0.5g of each plant extract was separately dissolved in 15ml of 95% ethanol and filtered. To 3 ml of each of the filtrate in a test tube, 1 ml of 0.5M potassium hydroxide was added. Development of a dark yellow colour confirmed the presence of flavonoids in the extract compounds (Appendix VI).

3.10.9 Test for gums and mucilage

Approximately 5 ml of each extract of 1mg/ml concentration was added to 5ml of absolute ethanol slowly with continuous mixing. Precipitate formation of gelatinous precipitate was an indication of presence of gums in the respective plant extract **(**Ruskin *et al.,* 2014)

3.10.10 Test for tannins

Two milliliters of each solvent extract (4mg/ml of respective solvent solution) was mixed with 5ml of distilled water and 3 drops of 5% ferric chloride ($FeCl_3$) added in separate test tubes. The formation of blue, blue green or black pigmentation would confirm presence of tannins (Ruskin *et al.,* 2014).

3.10.11 Test for resins

Acetone-H_2O Test (Ruskin *et al*., 2014) was used. Two milliliters of acetone was added into a test tube containing 100mg of each crude extract. To the mixture, 1ml of distilled water and shaken thoroughly. Turbidity showed the presence of resins in the respective extract.

3.10.12 Test for Phlabotannins

To 2ml of each crude extract of concentration 1mg/ml were added into separate test tubes followed by 2ml of 1% hydrochloric acid and the mixture heated to boiling. Formation of red precipitate was a confirmation of Phlobatannins presence (Harbone, 1984).

3.10.13 Test for terpenoids

This was done using Salkowski test (Ruskin *et al*., 2014). Briefly, 2mls of chloroform was separately added to 1ml of 1mg/ml of each solvent extract in a test tube. While holding the test tube in a slanting level, 3ml of concentrated sulphuric acid was added slowly down the wall of the test tube with a pipette resulting into two layered mixture. Appearance of a red-brown colour at the boundary between the layers confirmed terpenoids to be present.

3.10.14 Test for phenols

The phenols were tested following Ferric Chloride Test of Ruskin *et al*. (2014). One millitre of each extract solution in the respective solvent of extraction at a

concentration of 1mg/ml was mixed with 3ml of distilled water followed by 3 drops of 5% Ferric chloride. Appearance of a dark green colour showed that phenols were present.

3.10.15 Test for saponins

The Foam Test procedure of Brain and Turner, (1975) was used. About 0.5 g of the plant extracts were separately added into test tubes and 5 ml of water added into each fraction. The mixture was then shaken vigorously. Formation of foam which persists for about a quarter an hour indicated that saponins were the present in the respective extract.

3.10.16 Test for cardiac glycosides

About 200mg of every plant extract were constituted in 2 ml of its respective solvent of extraction in separate test tubes and 5ml of 0.5 M sulphuric acid added. The mixture was then heated to boiling with 5ml of 0.5 M sulphuric acid for 15 minutes in a water bath. The mixture was then allowed to cool before it was filtered and 5 ml of 1 M sodium hydroxide added to neutralize the acid. Two milliliters (2ml) of Benedict's solution was added and the contents of the test tube boiled in a water bath for two minutes. Appearance of brick red color in the mixture showed the presence of glycosides (Ruskin *et al*., 2014).

3.10.17 Test for volatile oils

To 1ml of every extract solution of concentration 1mg/ml was put in separate test tubes, 1ml of 90% ethanol were added followed by four drops of Iron (III) chloride solution. If a green color appeared volatile oils were confirmed present (Ruskin *et al*., 2014).

3.10.18 Test for emodols

Ruskin *et al*. (2014) procedure was employed whereby 200mg of each dry extract were added to 5ml of 25% ammonia solution in separate test tubes. The appearance of a red color in the solution is a confirmation of emodols present.

3.11 Statistical data analysis

The negative control wells containing parasite culture free from extracts and drugs were considered to have attained 100 % growth. The percentage inhibition per extract and standard drug concentration was determined by using the formula:

$$AV\%P = \frac{AV\ Ctrl - AV\ Treated}{AV\ Ctrl} \times 100$$

(WHO, 2001). One way and two way analysis of Variance (ANOVA) were used in comparing of average parasitemia levels between treatments. On the other hand, t-test was used to compare mean parasitemia levels between the two parasite strains. All $p<0.05$ values were considered statistically significant (Appendix VIII).

CHAPTER FOUR: RESULTS

4.1 Taxonomy of plants species investigated for anti-plasmodial activity

Three herbal medicine plants commonly used in treating malaria in Transmara west Sub County were identified and documented (Table 4.1). The plants were identified by botanical name, family and local name (Table 4.1).

Table 4.1: Taxonomy of plant species investigated on *P. falciparum*

Botanical Name	Plant family	Local name (s)	Part of plant
Rubia. cordifolia L.	Rubiaceae	Oloidodo	Leaf/stems
Harrisonia abyssinica Oliv.	Simaroubaceae	Ol-girigiri	Leaf/stems
Leucas calostachys Oliv.	Lamiaceae	Eng'enchemi	Leaf/stems

4.2 Extracts yields recovered from the three plant materials.

Crude extract yields recovered from plants under study during solvent extraction ranged between 6.6g (6.4% of the starting material) for *H. abyssinica* Oliv. hexane extract to 26.4g (17.6%) for the *R. cordifolia* water extract (Table 4.2).

Table 4.2: Quantities of extracts recovered from the extracts of the three medicinal plants.

Plant name	Plant part(s)	Powder Weight (g)	Yields of extracts (g) and solvents used		
			Water	Methanol	Hexane
R. cordifolia L.	Leaf	150	26.4	8.4	7.29
H. abyssinica Oliv.	Leaf	150	21.6	9.6	6.6
L. calostachys Oliv.	Leaf	150	22.8	10.2	8.7

The yields for all the three plants were as follows: Water>Methanol>Hexane. The plants generally produced extracts that were either brown or green in colour. The products were gummy or oily in texture. Aqueous extract obtained from *L. calostachys* and *R. cordifolia* were crystalline and black. A higher extract recovery was recorded in water extractions (Mean 23.53±2.04g) than in the other two solvents (Methanol, Mean=9.40±0.92g and Hexane, Mean=7.53±1.07g).

4.3 Growth characteristics of *P. falciparum* 3D7 and W2 strains

The parasite strains were maintained in a continous culture over a period of three months were monitored for parasitaemia growth every dayfor one week refreshing them but not diluting.the parasites attained exponential growth phase after the third day post incubation (Figure 4.1).

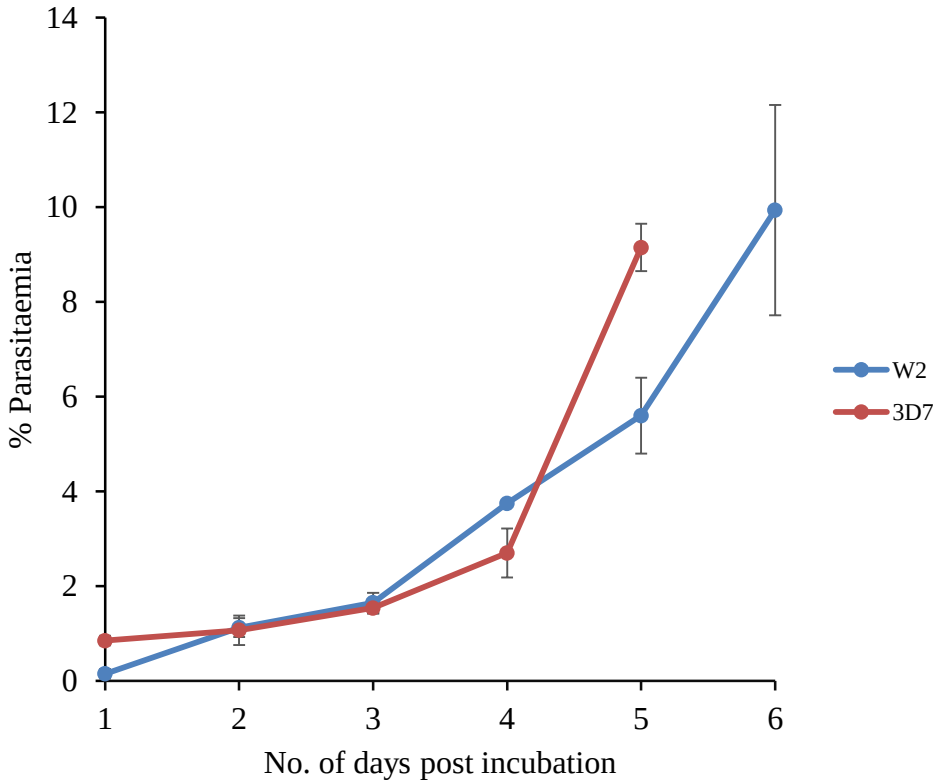

Figure 4.1: The Growth Patterns of *P. falciparum* 3D7 and W2 in culture. Each value is a mean of two observations.

The growth rate of the two *P. falciparum* strains did not differ significantly when compared using t-test. (t = 0.2967, p=0.7734) (Appendix VIII).

4.4 Chemotherapeutic effects of plants extracts on *P. falciparum* 3D7 and *P. falciparum* W2

In total, nine plants extracts were assayed for inhibitory effects against Chloroquine resistant (W2) and Chloroquine sensitive (3D7) strains of *P. falciparum* in a continuous culture. The level of efficacy of the extracts according to IC_{50} values was categorized as good (IC_{50}<5 µg/ml), moderate (IC_{50}>5 µg/ml to<50 µg/ml) and mild (IC_{50}>50 µg/ml (Muthaura *et al*., 2015) scale.

4.4.1 Effects of *L. calostachys* Oliv. extracts on the *P. falciparum* -W2 strain

Three solvent extracts of *L. calostachys* Oliv. (Water, Hexane and Methanol) were assayed for inhibitory effects against *P. falciparum* W2 (Chloroquine resistant) strain. Eight concentrations were used ranging from 50 to 0.4µg/ml for each extract. The parasitaemia levels in the negative control wells (mean, 4.54 ± 0.085) were significantly higher than that of water extract (Mean, 2.81± 0.228) (P<0.0001) as well as that in hexane extract treated wells (3.11± 0.14) (P<0.0001) and that of methanol (2.26± 0.05) (P<0.0001) indicating anti-plasmodial activity (Figure 4.2). Correlation analysis revealed that parasitaemia levels in solvents extract treated wells decreased with increasing concentration. The water extract of *L. calostachys* Oliv. induced significant decrease in levels of parasitaemia of *P. falciparum* W2 with increasing concentration of plant extracts (r = -0.752, p = 0.031). The mean parasitaemia levels in wells treated with hexane and methanol extracts did not change significantly with increase in extract concentration (r = 0.5714, and r = -0.5714), respectively. However, parasitaemia levels generally decreased with increase in extract concentration.

There was no significantly notable difference in parasitaemia levels between parasites treated with crude water and hexane extracts (p = 0.2815; t = 1.120; df = 14). Similarly, methanol crude extracts showed no significant difference in parasitaemia levels when evaluated against water crude extracts (p = 0.742; t = 1.930; df = 14). However, methanol crude extract showed significantly lower parasitaemia compared to hexane crude extract (p=0.0019; t=3.827; df = 14), implying a high antiplasmodial activity relative to that of hexane extract (Figure 4.2).

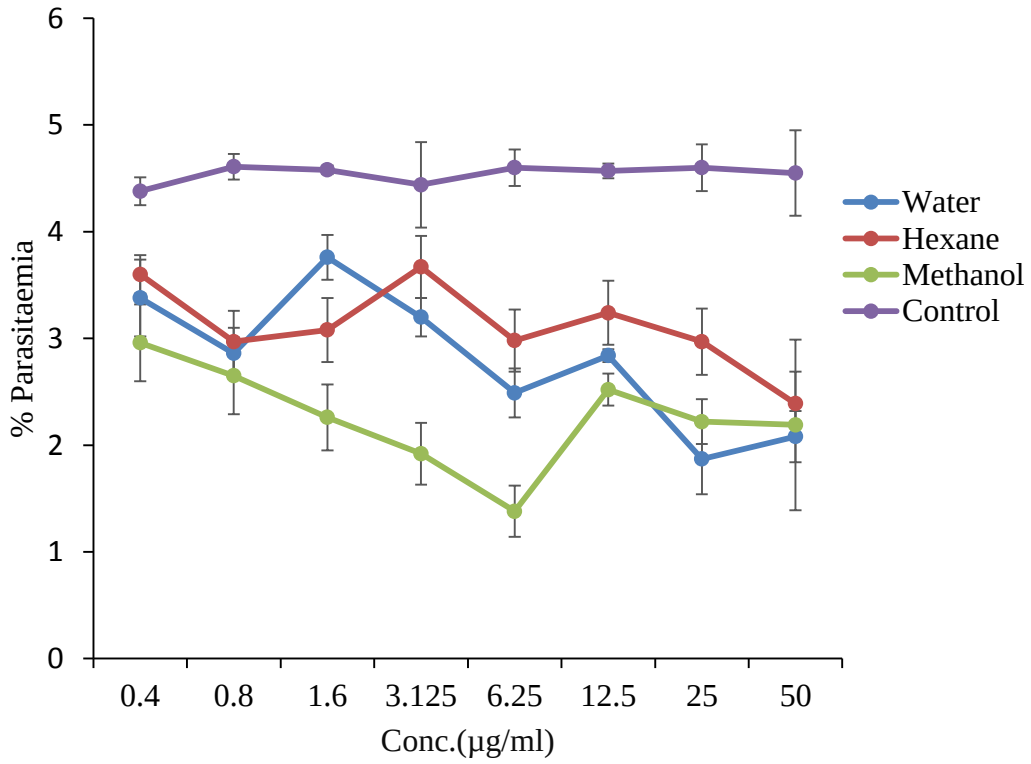

Figure 4.2: Parasitaemia levels of *P. falciparum*-W2 strain treated with extracts from *L. calostachys* Oliv.

4.4.2 Activity of extracts from *H. abyssinica* Oliv. on *P. falciparum* (W2)

The mean parasitaemia levels in negative control wells (4.54 ± 0.085) was significantly higher compared to parasites in wells treated with water (2.43± 0.55); hexane extract (mean, 3.15 ± 0.34) and methanol extract (mean, 2.23 ± 0.64).

(F=32.27, p<0.0001; Figure 4.3). There was no significant difference between the parasites treated with methanol extract and those treated with the crude water extracts (t=0.6501 p = 0.5261; Figure 4.3). Crude hexane extract had significantly higher parasitaemia levels than those of water (t=3.15, p=0.0076; Figure 4.3). Methanol extracts showed significantly lower parasitemia level when evaluated against hexane extracts (p= 0.00 31, t = 3.572; Figure 4.3). Using *H. abyssinica* Oliv. the result indicated that increase in extracts' concentration resulted into a significant decrease in levels of parasitaemia of *P. falciparum* W2 strain (r=-0.7856, p=0.0208; r-0.9051, p=0.0020 and r =-0.7414, p=0.0353) for water, hexane and methanol crude extracts respectively. This result showed that all the *H. abyssinica* were significantly effective on *P. falciparum* W2 strain.

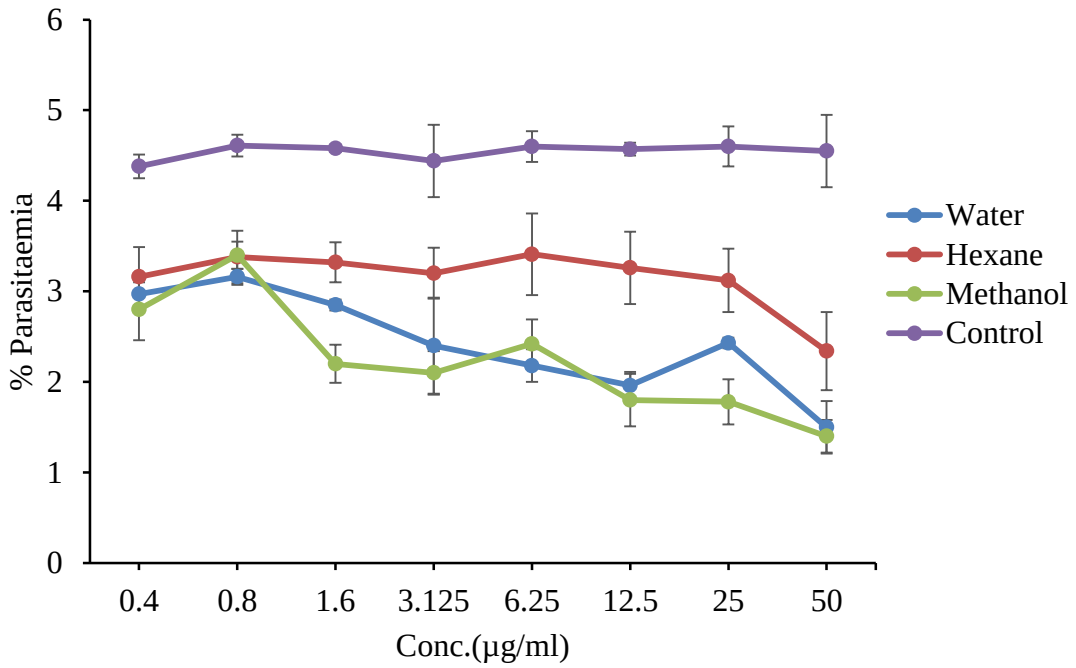

Figure 4.3: Parasitaemia levels for *P. falciparum*, W2 treated with extracts from *H. abyssinica* Oliv.

4.4.3 *In vitro* effects of extracts from *R. cordifolia* L. on the growth of *P. falciparum* W2.

Using water, hexane and methanol extracts from *R. cordifolia* L. against *P. falciparum* W2 strain, mean parasitemia of the negative control wells (4.54 ± 0.085); were significantly higher compared to parasites treated with water extracts (mean, 2.45 ± 0.63), hexane extracts (mean, 2.14 ± 0.54) and methanol extracts (Mean, 2.9 ± 0.43). ($F=88.74$, $P<0.0001$; Figure. 4.4). Correlation analysis of change in parasitaemia levels recorded in wells treated with *Rubia cordifolia* L. crude extracts against concentration was carried out. Increase in concentration of water extract of *R. cordifolia* L. resulted into significant decrease in levels of parasitaemia of *P. falciparum* W2 ($r= -0.7876$, $p= 0.0203$; Figure 4.4). Increase in concentration of methanol extracts did not produce significant reduction in parasitaemia levels of *P. falciparum* W2 ($r= 0.6750$, $p = 0.0662$ and $r = -0.4972$, respectively). On the other hand, increase in hexane crude extract concentration resulted in increase in parasitaemia levels. ($r =-0.675$, $p=0.066$). When evaluated against water and methanol extracts, hexane crude extracts treated wells recorded significantly lower parasitaemia levels ($t=2.205$, $p=0.0447$ and $t=2.277$, $p=0.0390$, respectively; Figure 4.4). Water extract treated wells did not significantly differ from methanol extracts in parasitaemia levels ($t=0.3481$, $p = 0.7329$; Figure4.4).

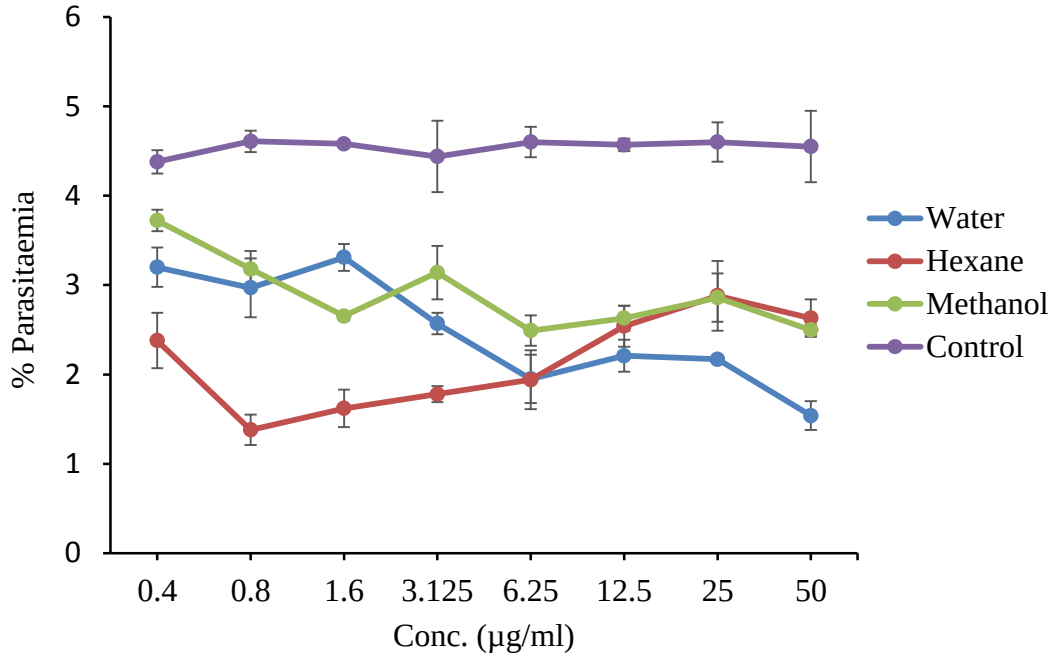

Figure 4.4: *In vitro* effects of extracts from *R. cordifolia* on growth *P. falciparum* W2.

4.4.4 *In vitro* activity of extracts from *L. calostachys* on *P. falciparum* 3D7

Mean percentage parasitaemia in control wells (mean, 4.76 ± 0.33) was shown to be more significantly higher than those treated with water extracts (mean= 2.595± 0.275), Hexane extracts (mean, 2.85 ± 0.14) and methanol extracts (mean, 2.1 ± 0.63). ($F=73.75$, $p<0.0001$). The result showed that, increase in concentration of water, hexane, and methanol extracts did not significantly reduce parasitemia levels of *P. falciparum* strain 3D7 ($r = -0.4752$, $p = 0.2340$; $r = 0.4358$, $P = 0.2805$ and $r=0.06823$, $p=0.87$) respectively. Unpaired t- test revealed no significant difference when crude water extract-treated wells were evaluated against methanol-extract treated wells ($t=2.032$, $p= 0.0615$) and also between water and hexane-extracts wells ($t=2.221$, $p= 0.0618$). Methanol crude extract-wells showed a significant difference when evaluated against hexane extract treated wells ($t=3.279$, $p= 0.0055$; Figure4.5).

Although not statistically significant, all *L. calostachys* crude extracts showed low parasitemia levels for *P. falciparum* 3D7 than *P. falciparum* W2. Parasitaemia levels generally decreased in the order Hexane>water>methanol extract treated wells for both strains indicating high antiplasmodial activity in methanolic extracts.

With aqueous extract of *L. calostachys* Oliv**.,** there was no significant difference in percentage parasitemia between *P. falciparum*, W2 (mean 2.81 ± 0.644) when evaluated against that of *P. falciparum* 3D7 (mean 2.595 ±0.275), (t = 1.02, P = 0.329).

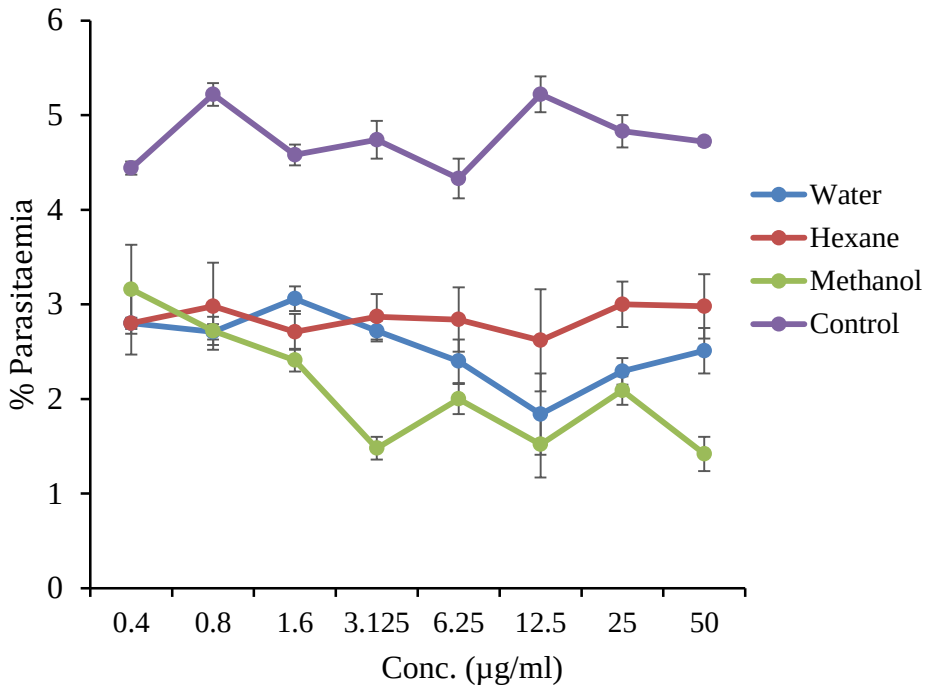

Figure 4.5: *In vitro* antiplasmodial effects of *L. calostachys* Oliv. extracts on *P. falciparum* 3D7 strain

Hexane extract (mean 3.11 ± 0.405) for *P. falciparum* of W2 strain showed no significant difference when compared with that of *P. falciparum* 3D7 strain (mean 2.29 ± 0.375), (t = 1.73, P = 0.11). Methanol extract of *L. calostachys* Oliv**.** also

produced no significant difference in parasitaemia levels when *P. falciparum* W2 strain was evaluated (mean 2.263 ± 0.479) against that of *P. falciparum* strain 3D7 (mean 2.100 ± 0.632), (t = 0.58, P = 0.572).

Table 4.3: Levels of Parasitemia of *P. falciparum* W2 and 3D7 treated with *L. calostachys* Oliv. extracts

	W2 strain	3D7 strain
Treatment	Mean ± SEM	Mean ± SEM
Aqueous	2.81± 0.23b	2.595 ± 0.1ab
Control	4.54 ± 0.03c	4.760 ± 0.12c
Hexane	3.111 ± 0.14b	2.850 ± 0.05b
Methanol	2.26 ± 0.17a	2.100 ± 0.22a
F-value	32.37	73.75
P-value	0.0001*	0.0001

Mean values in the same column represented by the same letters are not significantly different at p≤0.05. Mean separated by use of Tukeys HSD

4.4.5 Activity of extracts from *H. abyssinica* Oliv. on *P. falciparum* 3D7

Harrisonia abyssinica Oliv. crude extracts recorded significantly higher mean percentage parasitaemia in control wells (4.76 ± 0.33); than that obtained from the wells treated separately with water extracts (mean, 2.996±0.57); hexane extracts (mean, 3.29±0.38) and methanol extracts (mean, 2.31±0.57). (F=52.19, p<0.0001; Figure 4.6).

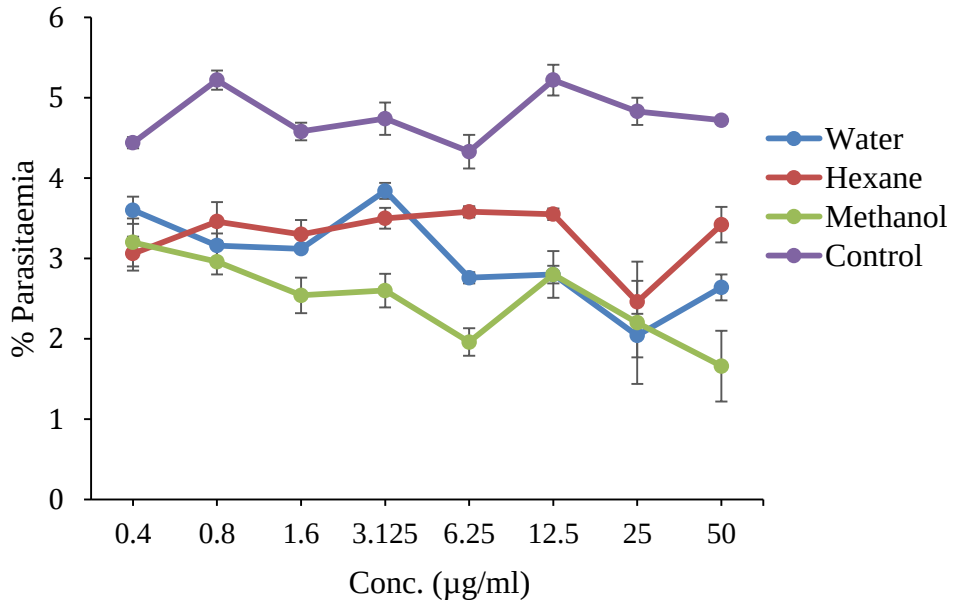

Figure 4.6: Percentage parasitaemia of *P. falciparum*, 3D7 parasites treated with extracts from *H. abyssinica* Oliv.

Water extract of *H. abyssinica* Oliv. showed no significant difference in parasitaemia levels of *P. falciparum* W2 strain (mean 2.43 ± 0.55) when compared with that of *P. falciparum* strain 3D7 (mean 3.00 ± 0.567), t = 2.01, P = 0.066.)

Hexane extract of *H. abyssinica* Oliv**.,** revealed no significant difference in parasitaemia levels of *P. falciparum* of W2 strain (mean 3.15 ± 0.34) when against that of *P. falciparum* strain 3D7 (mean 3.29± 0.38), (t = 0.79, P = 0.442). Similarly Methanol extracts showed no significant difference when its parasitaemia levels on *P. falciparum* W2 strain (mean 2.24 ± 0.64) were compared against those of *P. falciparum* 3D7 strain (mean 2.31 ± 0.57), t = 1.96, P = 0.07).

Table 4.4: Comparison of parasitemia Levels of *P. falciparum* W2 and 3D7 Strains exposed to *H. abyssinica* Oliv. crude extracts

	W2 strain	3D7 strain
Treatment	Mean ± SE	Mean ± SE
Aqueous	2.43 ± 0.20a	2.995 ± 0.2b
Control	4.541 ± 0.03c	4.76 ± 0.12c
Hexane	3.15 ± 0.12b	3.291 ± 0.1b
Methanol	2.24 ± 0.23a	2.31 ± 0.20a
F-value	38.10	37.15
P-value	0.0001	0.0001

Mean values in the same column indicated by the same letters are not significantly different at $p \leq 0.05$. Means were separated by Tukeys HSD.

Correlation analysis on the extract concentration and percentage levels of parasitemia recorded showed that, increase in concentration of methanol extract significantly resulted into a decrease in parasitemia levels of *P. falciparum* strain 3D7 (r = -0.787, P = 0.021). Increase in concentration of aqueous extraction also reduced the level of parasitemia of *P. falciparum* strain 3D7 (r = -0.616, P = 0.104). Decrease in the concentration of hexane extract from *H. abyssinica* Oliv. resulted into a significant decrease in the level of parasitemia of *P. falciparum* train 3D7 (r = 0.190, P = 0.653).

4.4.6 Activity of extracts from *R. cordifolia* L. on *P. falciparum* 3D7

The three solvent extracts from *R. cordifolia* L. were assayed for inhibitory effects on the growth of *P. falciparum* 3D7 at eight serial dilutions. Mean percentage parasitaemia in control wells (Mean, 4.76 ± 0.3273) was significantly higher when compared with those treated separately with extracts from Water (Mean, 2.41±0.27), Hexane (Mean 2.07±0.61) and Methanol extracts from *R. cordifolia* L. (Mean, 2.42 ± 0.32). (F= 88.19, P<0.0001; Figure 4.7) indicating that all the three *R. cordifolia* L, crude extracts possessed antiplasmodial activity.

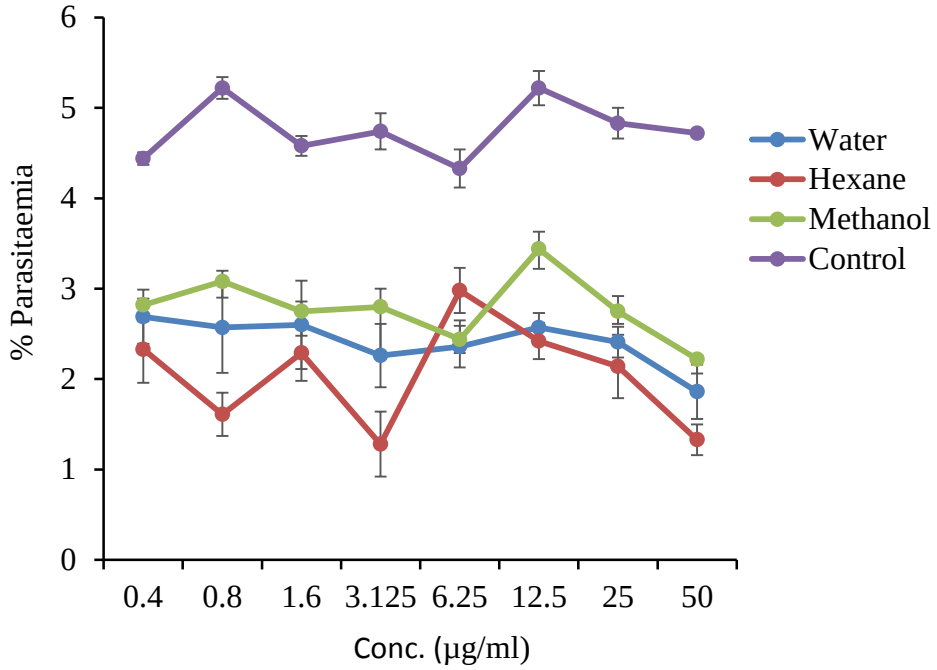

Figure 4.7: Average parasitaemia of *P. falciparum* 3D7 parasites treated with extracts from *R. cordifolia* L.

The hexane extract showed the highest level of inhibition of development of parasitaemia followed by the methanol extracts and then water.

Table 4.5: Levels of parasitemia of *P. falciparum* (W2) and (3D7) treated with *R. cordifolia* L. solvent extracts

	W2 strain	3D7 strain
Treatment	Mean ± SE	Mean ± SE
Aqueous	2.49 ± 0.22a	2.42 ± 0.09a
Control	4.53± 0.10b	4.76 ± 0.12c
Hexane	1.84± 0.19c	2.05 ± 0.21a
Methanol	2.90 ± 0.15a	2.79± 0.13b
F-value	51.17	88.19
P-value	0.0001	0.0001

The mean values occurring in the same column shown by similar letter symbols are not significantly different at $p \leq 0.05$. Means were separated using Tukeys HSD test.

4.4.7 Control treatment for the two strains of *P. falciparum*

There was no significant difference in parasitemia levels of untreated negative control for *P. falciparum*, W2 (mean=4.54 ± 0.086) when compared against that of *P. falciparum* strain 3D7 (4.76 ± 0.072) (t =1.830, p=0.1099). Though no statistically significant difference was noted between CQ and ATM treated parasites, *P. falciparum* W2 CQ treated wells generally had higher parasitaemia levels than ATM treated wells (Figure 4.8).

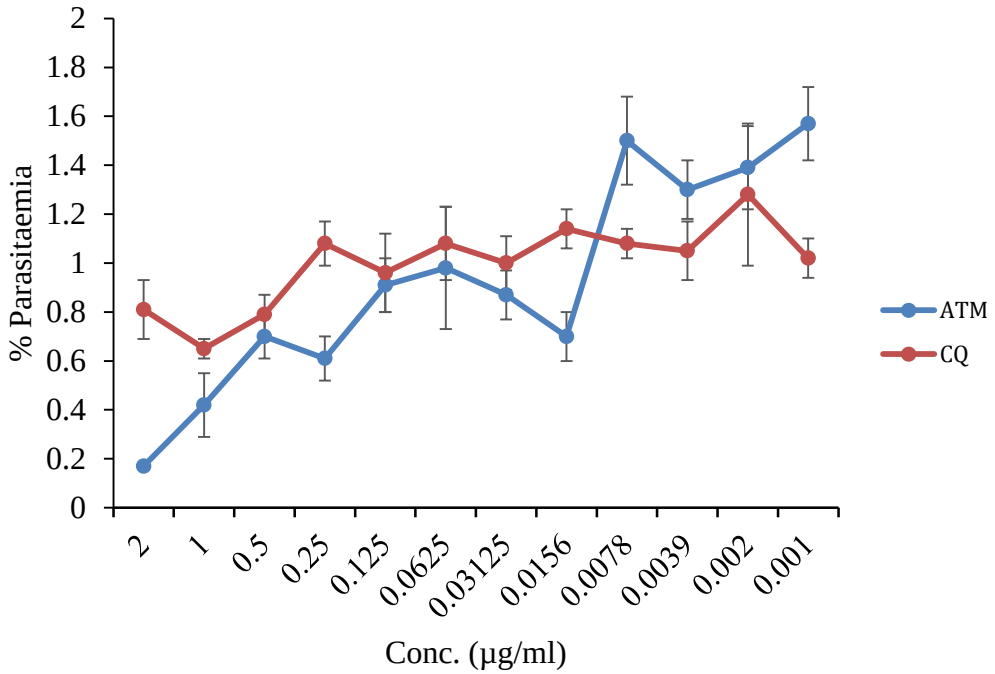

Figure 4.8: Levels of Parasitemia of *P. falciparum* W2 strain at different concentrations of CQ and ATM

All extracts as well as the standard control drugs suppressed the growth of both strains of *Plasmodium falciparum* (W2 and 3D7) in a concentration dependent manner.

4.5 IC_{50} Values for the 9 crude extracts and the standard control drugs

Table 4.6 shows IC_{50} values obtained for the 9 plant extracts assayed for *in vitro* inhibitory effects against chloroquine resistant *P. falciparum* (W2) and chloroquine

sensitive *P. falciparum* (3D7) respectively. IC_{50} values for *P. falciparam* W2 strain were 0.1294 µg/ml and 0.03043 µg/ml for CQ and ATM respectively. *P. falciparum* 3D7 strain also gave low IC_{50} values with CQ and ATM being 0.06488 µg/ml 0.005127 µg/ml for W2 and 3D7 respectively.

Out of the nine extracts tested, 3 displayed high antiplasmodial activity against 3D7 strain with IC_{50} values (2.1-4.457 µg/ml) while the rest 6 extracts showed moderate activity (5.477-39.02 µg/ml). High antiplasmodial activity was displayed by methanol extract of *L. calostachys* Oliv. (3.704 µg/ml), methanol extract of *R. cordifolia* L. (1.23 µg/ml), hexane extract of *Rubia cordifolia* L. (0.5517 µg/ml) and methanol extract of *H. abyssinica* Oliv. (2.682 µg/ml).

The methanol extract of *L. calostachys* Oliv. Showed the highest inhibitory effect on both *P. falciparum* 3D7 and *P. falciparum* W2 strains (IC_{50} = 1.815 µg/ml and 3.704 µg/ml) respectively. All tested extracts of *L. calostachys* Oliv. were shown to be more effective against *P. falciparum*, 3D7 (CQ sensitive) (1.815 µg/ml 2.75 µg/ml and 32.19 µg/ml) than the resistant strain (W2) (3.704 µg/ml, 5.288 µg/ml and 39.03 µg/ml). All tested extracts from *H. abyssinica* Oliv. and *R. cordifolia* L. showed higher inhibitory effects on CQ-resistant strain (W2) than the CQ- sensitive 3D7 strain as shown by higher IC_{50} values for the two plants extracts on *P. falciparum* 3D7 strain (Table 4.6). However, the water extract of *H. abyssinica* Oliv. showed almost the same moderate activity against W2 and 3D7 (IC_{50}=6.106 µg/ml and IC_{50}=6.140 µg/ml respectively). The methanolic extract of *H. abyssinica* Oliv. had a high activity against W2 than that of *L. calostachys* Oliv. IC_{50} 2.682 µg/ml and 5.262 µg/ml respectively.

All the tested extracts of *R. cordifolia* L. generally exhibited the highest inhibitory effects on both *P. falciparum*, W2 (CQ resistant) and *P. falciparum*, 3D7 (CQ sensitive) as compared to the other two plants. *Rubia cordifolia* L. hexane extract exhibited the highest anti-plasmodial activity (IC_{50}=0.5517 µg/ml) against chloroquine resistant strain, W2.

Table 4.6: Summary of IC_{50} values for the Plant extracts and control drugs on *P. falciparum* (W2) and 3D7 strains (µg/ml)

Plant/drug	Extract	IC_{50} values µg/ml(W2)	IC_{50} values µg/ml (3D7)
L. calostachys Oliv.	Water	5.288	2.75
	Hexane	39.03	32.19
	Methanol	3.704	1.815
H. abyssinica Oliv.	Water	6.106	6.140
	Hexane	30.12	39.04
	Methanol	2.682	5.262
R. cordifolia L.	Water	5.348	17.34
	Hexane	0.5517	2.747
	Methanol	1.231	3.528
CQ		0.1294	0.03043
ATM		0.06488	0.005127

CQ-Chloroquine ATM- Artemether

Chloroquine and artemeter functioned well as positive controls as indicated by their low IC_{50} values observed. Chloroquine had IC_{50} values 0.03043 µg/ml and 0.1294 µg/ml respective against chloroquine sensitive and resistant strains of *P. falciparum*.

On the other hand artemeter had IC_{50} values of 0.005127 µg/ml and 0.06488 µg/ml respectively, against the CQ sensitive and resistant *P. falciparum* strains.

4.6 Overall comparison of efficacies of plant extracts on *P. falciparum*, 3D7 (CQ sensitive) and *P. falciparum*, W2 (CQ resistant).

A paired sample t-test was used in comparing plant extracts activity on sensitive *P. falciparum* 3D7 strain and plant extract activity on chloroquine resistant *P. falciparum* W2 strains. The analysis revealed no significant difference in parastaemia levels between *P. falciparum*, 3D7 strain (mean 3.135 ±0.111) and *P. falciparum*, W2 (strain mean 3.095 ± 0.102), t = 0.79, P = 0.434).

To establish the effect of concentration and plants on the levels of *P. falciparum* W2 and 3D7 strains, two way analysis of variance (ANOVA) was used. Analysis of variance revealed no significant difference in concentration (F = 0.55, P = 794), plants extracts (F = 0.18, P = 0.835) and interaction of the two factors (F = 0.17, P = 1.000) on the levels of W2 percentage parastemia. Comparing the effect of different plant extracts using different solvents, there was a significant interaction effect on the level of parastemia of *P. falciparum* W2 strain (F = 5.55, P = 0.0001).

There was no significant difference in extracts concentration (F = 0.50, P = 0.830), plants extracts (F = 0.72, P = 0.490) and interaction of the two factors (plant and extract concentration) (F = 0.15, P = 1.000) on the other hand, parasitaemia levels of *P. falciparum* 3D7. Using different solvents for the different plants, the results showed that there was a significant interaction effect on the percentage parastemia of *P. falciparum* 3D7 (F = 6.75, P = 0.0001). Methanol extract was the most effctive solvent since its extract recorded the lowest parasitaemia levels (Table 4.7).

Table 4.7: **Mean levels of parastemia after plant extraction using the solvents on *P. falciparum* W2 and 3D7 strain.**

Solvent	Strain W2 % parastemia	Strain 3D7 % parastemia
Aquoeus	2.577 ± 0.124a	2.65 ± 0.10a
Control	4.536 ± 0.06b	4.76 ± 0.07b
Hexane	2.80 ± 0.13a	2.73 ± 0.13a
Methanol	2.465 ± 0.120a	2.397 ± 0.121a

Mean % parastemia ± SE in same collumn denoted using same letter symbols are not significantly different $p \leq 0.05$.

4.7 Phytochemical analysis of the crude plant extracts

4.7.1 *L. calostachys* Oliv. extracts

Qualitative phytochemical analysis revealed the presence of quinones, volatile oils, carbohydrates, phenols, saponins, fixed oils, flavonoids, tanins, and reducing sugars in methanol fraction of *L. calostachys* Oliv. (Table, 4.8). Water extracts contained fewer phytochemicals: carbohydrates, terpenoids, saponins, flavonoids, tannins and reducing sugars. On the other hand, hexane extract tested positive for presence of quinones, alkaloids, resins, terpenoids, fixed oils and flavonoids. The results showed that water and methanol extracts contained carbohydrates, phenols, saponins, flavonoids, tannins and reducing sugars in common. Alkaloids, resins, terpenoids, flavonoids were unique to hexane extract.

Table 4.8: Phytochemicals present in *L. calostachys* Oliv. Crude extracts

No.	Test	Aqueous	Hexane	Methanol
1	Acids	-	-	-
2	Betacyanins	-	-	-
3	Quinones	-	+	+
4	Volatile oils	-	-	+
5	Carbohydrates (Benedicts test)	+	-	+
6	Alkaloids (Hager's reagent test)	-	+	-
7	Aminoacids	-	-	-
8	Proteins	-	-	-
9	Resins	-	+	-
10	Phlabotannins	-	-	-
11	Terpenoids	-	+	-
12	Phenols	+	-	+
13	Saponins (foam test)	+	-	+
14	Fixed oils	-	+	+
15	Flavonoids (Ferric chloride test)	-	+	-
16	Flavonoids (Alkaline reagent test)	+	-	+
17	Tannins	+	-	+
18	Cardiac glycosides (Keller Kleen test)	-	-	-
19	Reducing sugars	+	-	+
20	Emodols	-	-	-

+ = Presence of bioactive compound; - = Absence of bioactive compound.

4.7.2 Phytochemical analysis of *H. abyssinica* Oliv. crude extracts

The results indicated the presence of acids, quinones, carbohydrates, alkaloids, terpenoids, phenols, flavonoids, tannins, cardiac glycosides, and reducing sugars In methanol crude extract. Water extract contained acids, betacyanins, quinones, carbohydrates, terpenoids, flavonoids and tannins and reducing sugars. Hexane extracts contained volatile oils, alkaloids, resins, terpenoids and flavonoids. All the three solvent extracts tested positive for flavonoids (Table 4.9).

Table 4.9: **Phytochemicals detected in *H. abyssinica* Oliv. Crude extracts**

No.	Test	Aqueous	Hexane	Methanol
1	Acids	+	-	+
2	Betacyanins	+	-	-
3	Quinones	+	-	+
4	Volatile oils	-	+	-
5	Carbohydrates (Benedicts test)	+	-	+
6	Alkaloids (Hager's reagent test)	-	+	+
7	Aminoacids	-	-	-
8	Proteins	-	-	-
9	Resins	-	+	-
10	Phlabotannins	-	-	-
11	Terpenoids	+	+	+
12	Phenols	-	-	+
13	Saponins (foam test)	-	-	-
14	Fixed oils	-	-	-
15	Flavonoids (Ferric chloride test)	+	-	+
16	Flavonoids (Alkaline reagent test)	+	+	+
17	Tannins	+	-	+
18	Cardiac glycosides (Keller Kleen test)	-	-	+
19	Reducing sugars	+	-	+
20	Emodols	-	-	-

+ = Presence of bioactive compound; - = Absence of bioactive compound.

4.7.3 Phytochemistry of *R. cordifolia* L. crude extracts

Methanolic extract of *R. cordifolia* was shown to contain quinones, volatile oils, carbohydrates, alkaloids, proteins, terpenoids, fixed oils, flavonoids, tannins, cardiac glycosides and reducing sugars. Aqueous extract contained betacyanins, quinones, carbohydrates, proteins, terpenoids, flavonoids and tannins while hexane extract contained quinones, volatile oils, alkaloids, resins, terpenoids, fixed oils, flavonoids

and cardiac glycosides. The tests showed that all the three solvent extracts had quinones, terpenoids and flavonoids in common (Table 4.10**).**

Table 4.10: Bioactive compounds in *R. cordifolia* L. Crude extracts

No.	Test	Aqueous	Hexane	Methanol
1	Acids	-	-	-
2	Betacyanins	+	-	-
3	Quinones	+	+	+
4	Volatile oils	-	+	+
5	Carbohydrates (Benedicts test)	+	-	+
6	Alkaloids (Hager's reagent test)	-	+	+
8	Proteins	+	-	+
9	Resins	-	+	-
10	Phlabotannins	-	-	-
11	Terpenoids	+	+	+
12	Phenols	-	-	-
13	Saponins (foam test)	-	-	-
14	Fixed oils	-	+	+
15	Flavonoids (Ferric chloride test)	+	+	+
16	Flavonoids (Alkaline reagent test)	-	-	+
17	Tannins	+	-	+
18	Cardiac glycosides (Keller Kleen test)	-	+	+
19	Reducing sugars	-	-	+
20	Emodals	-	-	-

+ = Presence of bioactive compound; - = Absence of bioactive compound.

CHAPTER FIVE: DISCUSSION, CONCLUSSION AND RECOMMENDATION

5.1 Discussion

5.1.1 Crude plant extracts yields of different solvent extracts of the plants

The three plants were extracted using water, methanol and hexane. Water was used so as to replicate the practice by herbalists (Were *et al.*, 2010). Methanol was selected due to its polarity that closely resembles that of water. Furthermore, some herbal medicines are made using ethanol, an alcohol obtained from fermented honey and beverages such as maize (Muthaura *et al.*, 2015). Furthermore, because of its intermediate polarity, the chemical composition for methanolic extracts serve as reference in phytochemical analysis. On the other hand, the choice of hexane was informed by the scientific fact that organic solvents dissolve and extract non polar compounds. In the current study, water as a solvent yielded more extracts (ranging between 14.4%-17.6%) followed by those of methanol extracts (5.6%-6.4%) while hexane yielded the lowest and ranged between 4.4 and 5.8%. This can be accounted for by the fact that that diverse solvents possess varied solubility capabilities for different phytochemical components (Marjorie, 1999). The results of the present study are in agreement with those of Muthaura *et al*., (2015) that recorded high water extract yields than hexane, methanol and ethyl acetate for medicinal plants from Kwale County, Kenya.

5.1.2 Growth characteristics of plasmodium falciparum 3D7 and W2 strains

The two *Plasmodium falciparum* strains used in this study were cultivated and used to determine *in vitro* chemotherapeutic effects of plant extracts. *Plasmodium falciparum* was chosen based on the fact that it causes up to 99% of the malaria cases worldwide and accounts for most deaths (WHO, 2017). Besides, it has shown rapid development of drug resistance to almost all conventional antimalarial drugs

(Greenwood *et al*., 2011). The two strains grew in culture to attain the highest parasitaemia of 10% between 4th and 7th day post incubation. The parasites were exposed to the extracts when they were at exponential phase of growth so that a reversal or change in growth rate thereafter could be attributed to the effects of the extract (Were *et al.*, 2010).

5.1.3 *In vitro* antiplasmodial effects of plant Crude extracts against *P. falciparum* 3D7 and W2 and their IC_{50} Values.

Leucas calostachys Oliv. methanol extract displayed high efficacy against both *P. falciparum* (3D7) and (W2) strains (IC_{50}=1.815 µg/ml and 3.704 µg/ml), respectively. All the extracts from *L. calostachys* Oliv. that were tested had higher activity against the CQ sensitive strain of *P. falciparum* (3D7) (IC_{50}, 1.815µg/ml, 2.75µg/ml and 32.19µg/ml) than the resistant strain (W2) (IC_{50}, 3.704µg/ml, 5.288µg/ml and 39.03µg/ml) indicating possible cross resistance in the compounds present with Chloroquine. These findings are in line with those recorded by Nyambati *et al*., (2015) that showed *L. calostachys* Oliv. to be active against *Plasmodium knowlesi*.

The *in vitro* antiplasmodial activity observed in this study generally showed methanol extracts of *L. calostachys* and *H. abyssinica* to be more effective as compared to those of hexane crude extracts. This can be explained by the fact that active polar phytochemical components are not extracted by hexane due to polarity differences. The result therefore agreed with those reported by Tona *et al*., (1999) which showed biologically active components to be more in methanol than in water extracts. On the hand, *R. cordifolia* hexane extract generally displayed a higher antiplasmodial activity than water and methanol extracts. This can be accounted for

by the fact that active lipophilic phytochemicals are not extracted by water and methanol. These findings are is in agreement with those reported by (Were *et al.*, 2010) that showed non- polar compounds to be more active on *P. knowlesi.*

Out of the nine extracts tested, 4 displayed high antiplasmodial activity against 3D7 strain with IC_{50} values (1.815-3.528 µg/ml) while the rest (5 extracts) showed moderate activity (5.262-39.02 µg/ml). High antiplasmodial activity was recorded in water and methanol extracts of *L. calostachys* Oliv. in addition to those of methanol and hexane extracts of *R. cordifolia* L. (IC_{50} =2.75, 1.815, 2.747µg/ml and 0.5517 µg/ml), respectively. The other extracts were moderately active as indicated by IC_{50} values that ranged between 5.262 µg/ml and 39.04 µg/ml. On the other hand, 4 crude extracts displayed high antiplasmodial activity against *P. falciparum*, W2 with $IC_{50} \leq 5$ µg/ml while the rest 5 extracts showed moderate activity (5 µg/ml$\geq IC_{50} \leq$50 µg/ml). High antiplasmodial activity was displayed by methanol extracts of *L. calostachys* Oliv. (3.704 µg/ml) and *R. cordifolia* L. (1.231 µg/ml) and hexane extract of *Rubia cordifolia* L. (0.5517 µg/ml) and methanol extract of *H. abyssinica* Oliv. (IC_{50} =2.682 µg/ml) while the other extracts displayed moderate activity ranging from 5.28 µg/ml-39.03 µg/ml. this can be accounted for by the fact that various phytochemicals have varied degree of solubility in different solvents according to level of solvent polarity. The present study therefore was in agreement with Gessler *et al*. (1994) who indicated high antiplasmodial activity (IC_{50} =0.38 µg/ml) for *Cissampelos mucronata* on KI strain. Other studies with similar findings include that of Muregi *et al*. (2004) on *Clerodendrum myricoides* ($IC_{50,}$ 4.0µg/ml on V1/S strain; *Maytenus senegalensis*, IC_{50}=3.9 µg/ml on 3D7strain) (Eltahir *et al*., 1999) and *Zanthoxylum chalybeum* (IC_{50}=8.1 µg/ml) (Bbosa *et al.*, 2014).

In some cases the chloroquine resistant strain (W2) appeared more responsive to the crude extracts as compared to susceptible one (3D7). For instance W2 strain was twice as susceptible as 3D7 strain to *H. abyssinica* Oliv. methanol extract ($IC_{50,}$ 2.682 µg/ml and 5.262 µg/ml) respectively, while *R. cordifolia* hexane extract was more than twice as active against W2 than 3D7 ($IC_{50,}$ 0.5517 µg/ml and 2.722 µg/ml) respectively. This suggests possible absence of cross resistance in the phytochemicals present in the extracts with chloroquine most likely as a result of dissimilarities in the manner of action of their molecules. The results suggest that phytochemicals from these plants could be potential alternatives of reversing chloroquine resistance. Results of the study also agreed with those reported by Masila, (2014) which showed water-methanol crude extract of *H. abyssinica* Oliv. to have IC_{50} values of 5.6 and 4.4 µg/ml against sensitive *P. falciparum*, D6 strain and resistant *P. falciparum* Chloroquine resistant, W2 strain, respectively.

In some cases, previous researchers reported different antiplasmodial activities for the same plants as those investigated under this study. For example, Jeruto *et al*., (2015) found water and methanol extracts of *Leucas calostachys* Oliv. collected from Nandi County to be less effective on CQ sensitive D6 strain (IC_{50}>100 µg/ml and 88.5 µg/ml), respectively while Muregi *et al*. (2004) recorded IC_{50} values of 66±2.1; 87.7±1.77 and IC_{50}>100µg/ml) for *L. calostachys* Oliv. hexane, methanol and water extracts, respectively against chloroquine sensitive *P. falciparum*, K39 strain. These observations can be accounted for by the fact that the concentrations and numbers of bioactive phytochemicals contained in plant extracts vary based on variety, place of origin, environmental conditions of processing, nature of the solvent of extraction as well as intra-species variations (Muregi *et al*., 2004).

Chloroquine and artemether functioned well as positive control. *Plasmodium falciparum*, W2 had a higher IC_{50} and parasitaemia levels than 3D7, indicating chloroquine resistance in W2.

5.1.4 Phytochemicals composition of the plant extracts

The present study, showed that *L. calostachys* Oliv. contained quinones, volatile oils, carbohydrates, terpenoids, phenols, saponins, fixed oils, flavonoids, reducing sugars and tanins in methanol extraction. Aqueous extract contained carbohydrates, terpenoids, flavonoids, tanins, saponins and reducing sugars whereas hexane extracts of the same plant contained fewer compounds namely Quinones, alkaloids, resins, terpenoids, flavonoids and fixed oils. The present study results agree with those recorded by Okach *et al.,* (2013) who reported saponins, glycosides, flavonoids, alkaloids and taninns in aqueous extract of *L. calostachys* Oliv. collected from Migori County.

Leucas calostachys Oliv. has been indicated for the treatment of headache and colds (Okello *et al*., 2010); Okach *et al.,* (2013) detailed widespread usage of its concoction, infusion and maceration for the treatment of stomachache, diarrhea, gastrointestinal diseases, constipation and cold in Uriri District. Previous works involving plants in Lamiaceae family including *Leucas aspera* reported strong analgesic characteristics of the plants attributed to their rich alkaloid composition **(**Suarez, 2003). This suggests a high probability that the extracts or the plants used in the current study that contain alkaloids could better be serving in relieving pain related to malaria due to their analgesic properties, in addition to direct antimalarial activity in traditional medicine practice.

Results of the present study also showed reducing sugars, tannins, flavonoids, alkaloids, terpenoids, phenols, cardiac glycosides, carbohydrates, acids and quinones to be present in methanol crude extract from *H. abyssinica* Oliv. On the other hand carbohydrates, terpenoids, reducing sugars, betacyanins, tanins, quinones and flavonoids were detected in water extracts. Fewer bioactive molecules were found present in hexane extracts namely: flavonoids, terpenoids, quinone, volatile oils, alkaloids and resins. This could be accounted for by the low solubility of the rest of the compounds in hexane being a non-polar solvent (Marjorie *et al.*, 1999). The findings of the current study agree with a previously documented study where a triterpenoid, Obacunone was isolated from *H. abyssinica* Oliv. The triterpenoid that was isolated attained a low IC_{50} values with sensitive D6 and resistant W2 *P. falciparum* strains (IC_{50} ≥4.76 µg/ml) (Masila, 2014). Members of the genus *Harrisonia* have also been shown to contain limonoids, chromones with a wide range of efficacy.

Phytochemical analysis of *R. cordifolia* L. revealed Quinones, terpenoids and cardiac glycosides to be present among other compounds. This was found to agree with the past studies that showed *R. cordifolia* L. to contain quinones, Iridoids, 6-methoxygeniposidic acid (Wu *et al*., 1991); and triterpenoids (Itokawa *et al*., 1989). Antiplasmodial activity observed for *R. cordifolia* L. in this study is attributable to the same compounds. However, this is the first report made on the antiplasmodial effects that R. *cordifolia* L. has on *P. falciparum* W2 and 3D7. The compounds have also been shown to possess hypotensive and analgesic properties (Younos *et al*., 1990). The plants in the genus *Rubiaceae* can therefore help in managing malaria

associated pain. These therefore justify using *R. cordifolia* L. for treating malaria in traditional herbal medicine.

The phytochemical tests conducted revealed that all the three plants investigated in this study tested positive for quinones, carbohydrates, alkaloids, terpenoids, flavonoids, tanins and reducing sugars. This is a strong signal that they have a potent therapeutic value particularly to those who use them. Natural quinones are said to possess naphtoquinone, anthraquinone and benzoquinone structures which closely resembles that of atovaquone, a known effective antimalarial drug (Basco *et al*., 1995).

Naphthoquinones obtained from *Kigelia pinnata* was determined to have a high antiplasmodial efficacy (IC_{50} = 0.002 μg/ml) (Weiss *et al*., 2000). Similarly, Xestoquinone, aquinone isolated from *Xestospongia sp.*, a marine sponge was shown to have a high inhibitory effect on plasmodial growth (IC_{50} = 3 μM) (Laurent *et al*., 2006) all confirming the antimalarial properties of quinones observed on the plants investigated in the present study.

5.2 Conclusions

From *in vitro* antiplasmodial activity studies;

i. The medicinal plants *L. calostachys* Oliv, *H. abyssinica* Oliv. and *R. cordifolia* L. demonstrated activity against CQ resistant *P. falciparum*, W2. strain. *R. cordifolia* L. hexane extract recorded the highest activity ($IC_{50,}$ 0.5517μg/ml) against W2 strain.

ii. The methanolic extracts of *L. calostachys* Oliv., *H. abyssinica* Oliv. and *R. cordifolia* L. extracts showed highest antiplasmodial activity (IC_{50},

1.815 µg/ml, 5.262 µg/ml and 3.528 µg/ml respectively) against drug sensitive *P. falciparum* 3D7 strain compared to resistant *P. falciparum,* W2. *Plasmodium. falciparum,* W2 was found to be generally more responsive to *H. abyssinica* Oliv. and *R. cordifolia* L. crude extracts than CQ sensitive *P. falciparum,* 3D7 while the reverse was true for *L. calostachys* Oliv. crude extracts.

iii. *L. calostachys* Oliv. *H. abyssinica* Oliv. and *R. cordifolia* L. contain bioactive molecules such as quinones, alkaloids, terpenoids and flavonoids being common among them to which antiplasmodial activity was attributable. In addition hexane extract of *R. cordifolia* L. contained organic compounds most likely volatile oils and fixed oils that could have been responsible for high antiplasmodial activity against W2 strain.

5.3 Recommendations

Based on this study, it is recommended as follows:

i. The plants studied demonstrated that they are potential source of useful antimalarial drugs. The study recommends their continued use in treating malaria particularly *H. abyssinica* which is more active on chloroquine resistant *P. falciparum* preferably with methanol as extraction solvent.

ii. Drug interaction studies to be done to determine synergistic effects of compounds present in these plants in combination with chloroquine with the aim of determining the possibility of reversing chloroquine resistance.

iii. Bioactive compounds present in these medicinal plants to be isolated, purified and characterized and separately investigated to obtain useful chemotherapeutic agents present in these plants.

5.4 Areas of further research

i. Future research should focus on establishing the *in vivo* antiplasmodial activities and cytotoxic properties of efficacious extracts from *R. cordifolia,* L. *calostachys* Oliv. and *H. abyssinica* Oliv.

ii. Future studies should carry out drug interaction studies to evaluate efficacy of the crude preparations and isolated pure compounds in combinations with chloroquine against CQ-resistant W2 strain to determine if CQ-resistance can be reversed.

iii. Efficacy of the crude extracts and their isolated phytochemicals to be evaluated against other disease causing micro-organisms.

5.5 Limitations of the study

The plants were harvested over the month of April which is a wet season in the region. With reasoning that phytochemical composition of the medicinal plants vary with season of harvest, age, ecological region among other factors, future studies should consider sampling the plants during different seasons.

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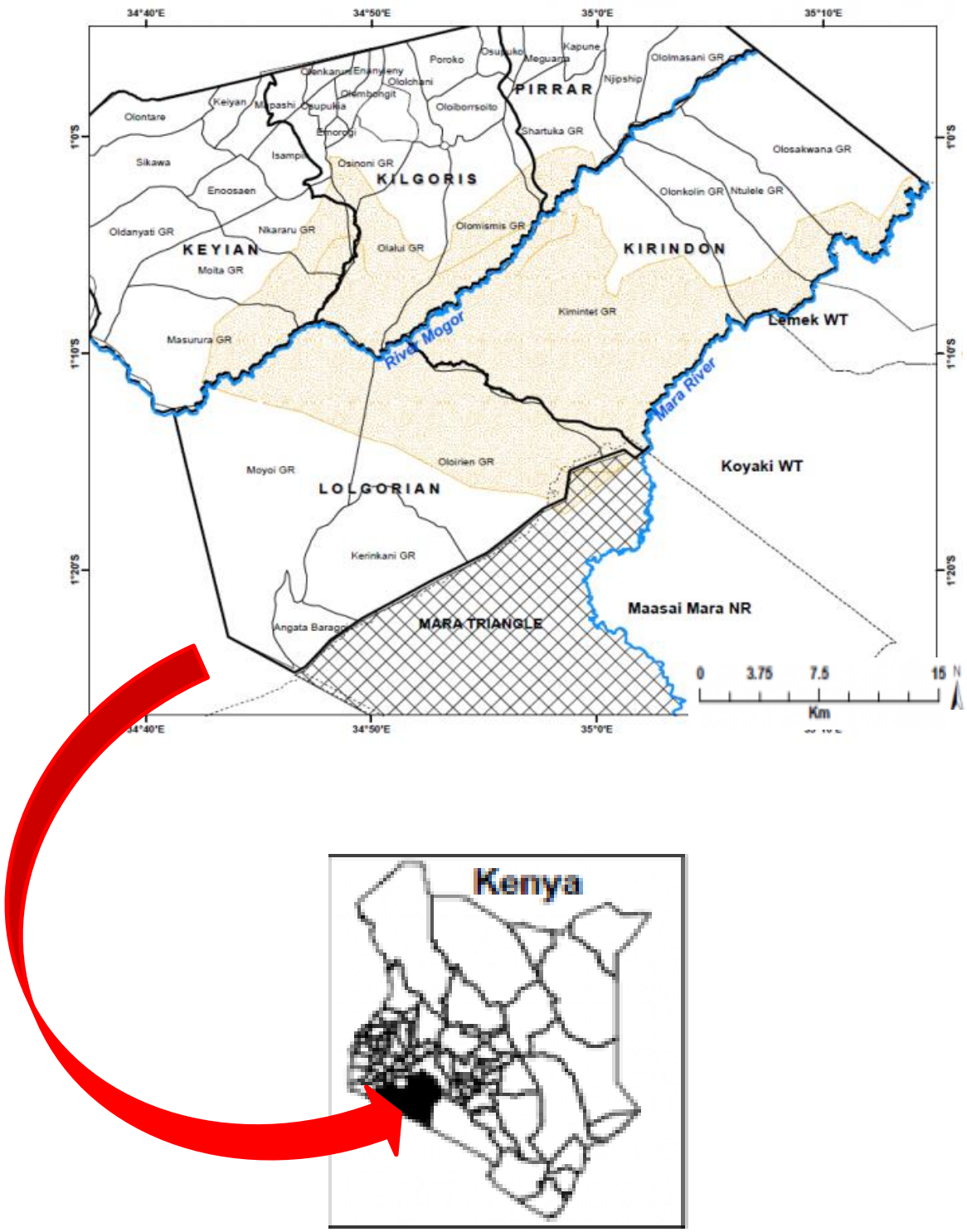
APPENDICES

APPENDIX I: Aerial part of *H. abyssinica* Oliv.

APPENDIX II: Aerial part of *L. calostachys* Oliv.

APPENDIX III: Aerial part of *R. cordifolia.* L.

APPENDIX IV: Map of Transmara West sub-county and its location in Kenya



APPENDIX V: Preparation of culture media and freezing mixture

a) Incomplete culture media

To prepared 50ml of incomplete culture media 1.9 ml of HEPES(3.8%) was added to 405.2 ml (90.4)% of RPMI (GIBCO) followed by 1.9ml (3.8%) of sterile 5% sodium bicarbonate. A half a mililitre (0.5ml) sterile 20% D-glucose solution (1%) was added and then 0.5ml (1%) of 200mM L-glutamine. 25µl of gentamycin was added to prevent microbial contamination. P^H of the mixture was adjusted to 7.2 and the mixture filtre sterized using 0.22µm filtre. The mixture was then stored at 4ºC till required for use. The procedure was aseptically carried out in class II biological bioseafetyhood.

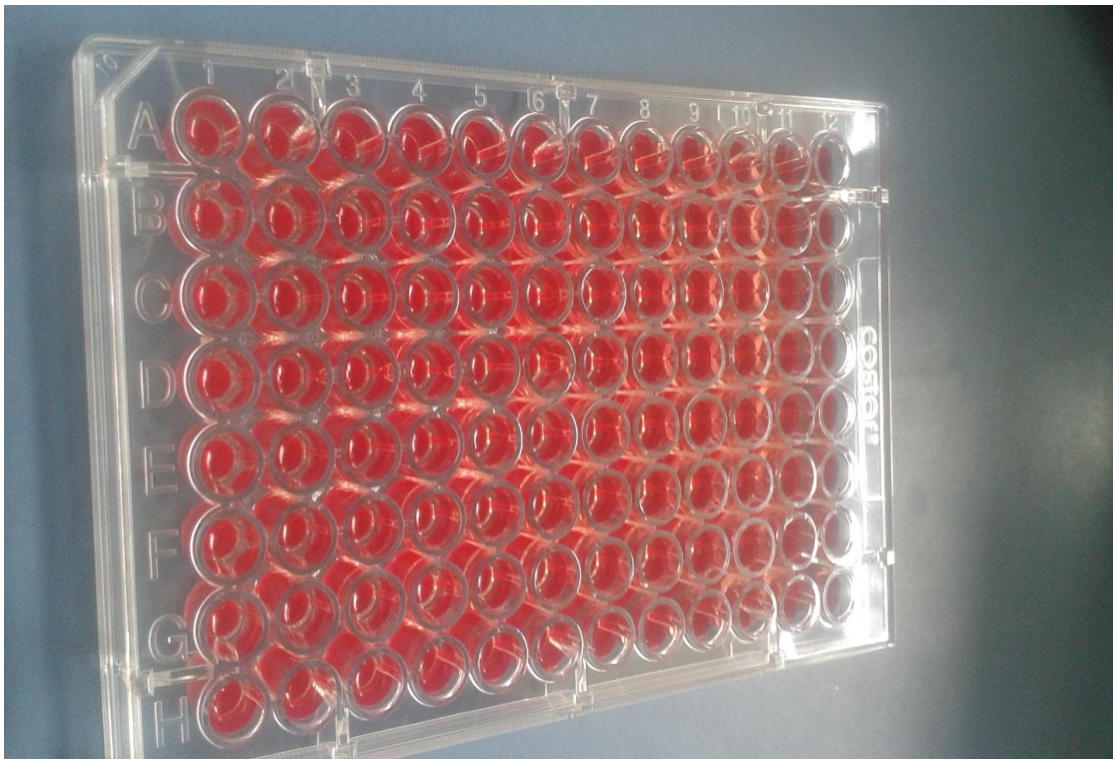
b) Complete culture media

To prepare 50 ml of complete culture media, 5ml (10%) of human serum was added to 45 ml (90%) of incomplete culture media prepared as above. The mixture was kept at 4°C and used within one week.

c) Freezing medium

The freezing medium used was prepared by mixing 28ml of of glycerol with 72ml of 4.2%sorbitol in normal saline. The mixture was filtre sterilized using 0.22µm filtres and then stored at 4ºC till required for use.

APPENDIX VI: 96-well flat bottomed culture plate used in the study



APPENDIX VII: Set up showing results for alkaline reagent phytochemical test for flavonoids.

KEY

1. *Rubia cordfolia* aqueous extract
2. *Rubia cordfolia* hexane extract
3. *Rubia cordfolia* methanol extract
4. *Leucas calostachis* aqueous extract
5. *Leucas calostachis* hexane extract
6. *Leucas calostachis* methanol extract
7. *Harisonia abyssinica* aqueous extract
8. *Harisonia abyssinica* hexane extract
9. *Harisonia abyssinica* methanol extract

APPENDIX VIII: Statistical analysis

a) Unpaired t-test of growth rate of *P. falciparum* W2 and 3D7

	Data Set-A
Table Analyzed	Data 1
Column B	3D7
vs.	vs.
Column A	W2
Unpaired t test	
P value	0.7734
P value summary	Ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.2967 df=9
How big is the difference?	
Mean ± SEM of column A	3.703 ± 1.483, n=6
Mean ± SEM of column B	3.062 ± 1.555, n=5
Difference between means	-0.6405 ± 2.158
95% confidence interval	-5.523 to 4.242
R squared (eta squared)	0.009689
F test to compare variances	
F, DFn, Dfd	1.091, 5, 4
P value	0.9593
P value summary	ns
Significantly different (P < 0.05)?	No

Ns= not significantly different at 95% confidence interval

b) Correlation matrix: Concentration of *L. calostachys*, aqueous, hexane, methanol, control on *P. falciparum* strain W2

Concentration	Aqueous	Hexane	Methanol	
Aqueous	-0.752			
	0.031			
Hexane	0.477	-0.411		
	0.232	0.311		
Methanol	-0.129	0.308	-0.771	
	0.761	0.459	0.025	
Control	-0.181	0.302	-0.639	0.660
	0.668	0.467	0.088	0.075

Cell Contents: Pearson correlation
P-Value

c) Correlation matrix: concentration of *L. calostachys*, aqueous, hexane, methanol, control on *P. falciparum* strain 3d7

Concentration	Aqueous	Hexane	Methanol	
Aqueous -	0.351			
	0.394			
Hexane	0.436	0.172		
	0.280	0.685		
Methanol	-0.558	0.530	0.027	
	0.151	0.177	0.950	
Control	-0.798	0.098	-0.401	0.463
	0.018	0.818	0.325	0.248

Cell Contents: Pearson correlation
P-Value

d) Correlation matrix: Concentration of *H. abyssinica*, aqueous, hexane, methanol, and control on *p. falciparum* strain w2.

	Concentration	Aqueous	Hexane	Methanol
Aqueous	-0.786			
	0.021			
Hexane	-0.905	0.662		
	0.002	0.074		
Methanol	-0.741	0.844	0.625	
	0.035	0.008	0.098	
Control	-0.711	0.643	0.889	0.459
	0.048	0.086	0.003	0.253

Cell Contents: Pearson correlation
P-Value

e) Correlations: Concentration of *H. abyssinica*, aqueous, hexane, methanol, control on *P. falciparum* strain 3D7

`	Concentration	Aqueous	Hexane	Methanol
Aqueous	-0.616			
	0.104			
Hexane	-0.190	0.467		
	0.653	0.243		
Methanol	-0.795	0.794	0.125	
	0.018	0.019	0.769	
Control	-0.798	0.069	-0.229	0.391
	0.018	0.870	0.585	0.339

Cell Contents: Pearson correlation
P-value

f) Correlation matrix: Concentration of *R. cordifolia*, Aqueous, Hexane, Methanol, Control on *P. falciparum* strain W2

	Concentration	Aqueous	Hexane	Methanol
Aqueous	-0.78			
	0.020			
Hexane	0.675	-0.571		
	0.066	0.139		
Methanol	-0.497	0.648	-0.140	
	0.210	0.082	0.742	
Control	-0.711	0.524	-0.269	0.142
	0.048	0.182	0.520	0.737

Cell Contents: Pearson correlation
P-Value

g) Correlation matrix: Concentration of *R. cordifolia*, Aqueous, Hexane, methanol, control on *P. falciparum* strain 3D7

	Concentration	Aqueous	Hexane	Methanol
Aqueous	-0.817			
	0.013			
Hexane	-0.354	0.526		
	0.390	0.181		
Methanol	-0.516	0.700	0.121	
	0.190	0.053	0.776	
Control	-0.798	0.836	0.619	0.489
	0.018	0.010	0.102	0.219

Cell Contents: Pearson correlation
P-Value

h) Paired T-test and CI: W2, 3D7

Paired T for W2 - 3D7

	N	Mean	St Dev.	SE Mean
W2	96	3.095	1.001	0.102
3D7	96	3.135	1.087	0.111
Difference	96	-0.0403	0.5035	0.0514

95% CI for mean difference: (-0.1424, 0.0617)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.79 P-Value = 0.434

i) Two-way ANOVA: W2 versus plants, concentrations

Analysis of Variance for W2

Source	DF	SS	MS	F	P
Plants	2	0.44	0.22	0.18	0.835
Concentration	7	4.67	0.67	0.55	0.794
Interaction	14	2.82	0.20	0.17	1.000
Error	72	87.32	1.21		
Total	95	95.24			

j) Two-way ANOVA: 3D7 versus plants, concentrations

Analysis of Variance for 3D7

Source	DF	SS	MS	F	P
Plants	2	2.04	1.02	0.72	0.490
Concentration	7	4.99	0.71	0.50	0.830
Interaction	14	3.06	0.22	0.15	1.000
Error	72	102.22	1.42		
Total	95	112.31			

k) Two-way ANOVA: W2 versus plants, solvent

Analysis of Variance for W2

Source	DF	SS	MS	F	P
Plants	2	0.440	0.220	0.96	0.388
Solvent	3	67.874	22.625	98.59	0.000
Interaction	6	7.649	1.275	5.55	0.000
Error	84	19.277	0.229		
Total	95	95.240			

l) Two-way ANOVA: 3D7 versus plants, solvent

Analysis of Variance for 3D7

Source	DF	SS	MS	F	P
Plants	2	2.044	1.022	5.31	0.007
Solvent	3	86.299	28.766	149.43	0.000
Interaction	6	7.799	1.300	6.75	0.000
Error	84	16.171	0.193		
Total	95	112.313			

m) ONE way ANOVA of the effect of *L. calostachys* extracts on *P. falciparum* W2

Descriptive					
ANOVA Table	SS	df	MS	P-Value	F-value
Treatment (between columns)	22.69	3	7.564	P<0.0001	32.37
Individual (between rows)	0.8006	7	0.1144		
Residual (random)	4.907	21	0.2336		
Total	28.4	31			
Bonferroni's multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Water vs Hexane	0.3013	1.246	No	ns	-1.005 to 0.4026
Water vs methanol	0.5475	2.265	No	ns	-0.1563 to 1.251
Water vs Control W2	-1.731	7.163	Yes	***	-2.435 to -1.027
Hexane vs Methanol	0.8487	3.512	Yes	*	0.1449 to 1.553
Hexane vs Control W2	-1.43	5.917	Yes	***	-2.134 to -0.7262
MeOH vs Control W2	-2.279	9.429	Yes	***	-2.983 to -1.575