INVESTIGATION OF *IN VITRO* ANTIPLASMODIAL ACTIVITIES OF *Carissa edulis, Azadirachta indica, Cassia siamea* AND *Harrisonia abyssinica* ON *Plasmodium falciparum*

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DECLARATION AND RECOMMENDATION

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DEDICATION

I dedicate this study to my uncle Professor Oduor Okello, my beloved wife Roseline Oduor, my sons Allan Victor Omondi and Fonfred Job Owino and my daughter Oduor Macreen Akoth.

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ABSTRACT

Malaria is caused by parasites in the genus *Plasmodium.* It is one of the leading causes of human morbidity and mortality in the sub-Sahara countries, Kenya included. The parasite is transmitted by *Anopheles sp* as a definitive host from one human host to another through bite. Five species of mosquito - borne *Plasmodium* parasites infect humans include *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesi*, and *Plasmodium falciparum.* Plant samples were collected from Masumbi village, Siaya County-Kenya. Botanical identification of the plant samples was done in the department of Biological Sciences, Egerton University and their voucher samples deposited in a departmental herbarium. Extraction, concentration, spotting and phytochemical analysis were carried out at the Department of Chemistry, Egerton University-Kenya. Phytochemical tests were done to determine the class of phytochemicals present in the methanolic crude extracts while brine shrimp cytotoxicity assay was carried out to predict the potential toxicity of the methanolic plant extracts. This study evaluated the phytochemicals, antiplasmodial effect, and cytotoxicity from extracts of *Carissa edulis* root barks, *Azadirachta indica* leaves, *Cassia siamea* stem barks and *Harrisonia abyssinica* root bark. Results of methanolic extracts of four different species of plants were analyzed for their phytochemical composition. Alkaloids, flavonoids, glycosides, saponins, steroids, tannins and terpenoids were detected in the extracts. This study established that extracts from the four medicinal plants, had plasmodial effects, however further studies are required to purify the seven identified phytochemicals for possible use in vivo.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-Based Combination Therapy
AMA1	Apical Membrane Antigen 1
BSC	Biosafety Cabinet
CMS	Complete Medium with Serum
CPDA-1	Citrate Phosphate Dextrose Adenine-1
CQ	Chloroquine Diphosphate
CQ-R	Chloroquine Resistant *Plasmodium falciparum* strains
CQ-S	Chloroquine Sensitive*Plasmodium falciparum* strains
3D7	Chloroquine Sensitive and mefloquine resistant strain of *P. falciparum*
DMSO	Dimethyl Sulfoxide
IC	Inhibitory Concentration
ITCZ	Intertropical Convergence Zone
KMIS	Kenya Malaria Indicator Survey
MDR	Multi Drug Resistance
MJ	Moving Junction
MQ-R	Mefloquine Hydrophosphate Resistance *Plasmodium falciparum* strain
MQ-S	Mefloquine Hydrochloride Sensitive *Plasmodium falciparum* strain
$NaH_2PO_4.H_2O$	Sodium Monobasic Phosphate
RF	Retention Factor
RFU	Relative Fluorescence Units
RON	Rhoptry Neck
RPMI	Roswell Park Memoria
SYBR	Synergy Brands
W2	Chloroquine Resistant And Mefloquine Sensitive Strains
WMR	World Malaria Report
WHO	World Health Organization
Q.R.	Quantity Required

CHAPTER ONE
INTRODUCTION

1.1 Background information

Malaria is caused by protozoan parasites of the genus *Plasmodium*. The parasites are transmitted by *Anopheles sp* as a definitive host from one human - being to another through abite. Five species of mosquito - borne *Plasmodium* parasites infect humans. These include *Plasmodium vivax, Plasmodium ovale, Plasmodium* malariae*, Plasmodium knowlesi*, and *Plasmodium falciparum.* The later causes the most virulent form of malaria, and the highest mortality rates in tropical countries. The disease caused by *Plasmodium falciparum*, if not properly treated and managed in time, there are always high possibility of the parasite developing resistance to drug, (Greenwood *et al.,* 2008). More than 40 % of the world population which is an estimate of 1.2 billion people is at high risk of transmission, (> 1 case per 1000 population). Half of this estimate lives in Africa region with 80 % of such cases being found in 13 countries including Congo, Nigeria, Ethiopia, Tanzania and Kenya, (World Health Organization, 2008).

More than 2.4 billion people from over 90 countries worldwide suffer from malaria with the highest disease burden reported in children in sub- Saharan African, (WHO, 2012). Studies of genomic diversity of *P. falciparum* had led to demography, global population structure and profile of natural selection, (Miotto, 2013). Report on genome sequencing and analysis of 825 *P. falciparum* collected from West Africa and South-east Asia, revealed an unexpected cluster of clonally propagated parasite subpopulations resistant to artemesinin, which is currently a key antimalarial drug, (Carlton *et al.,* 2008).

Currently, there is no approved drug without the history of drug resistance for malaria, yet drugs are indispensable components of malaria control programmes. Effective drug treatment of infected individuals has contributed significantly to the decline in malaria morbidity and mortality worldwide. However, the propensity or natural tendency for *P. falciparum* to develop resistance to anti-malarial drugs is troubling. Drug resistance has been reported for every existing approved anti-malarial drug or drug combinations, (Dondorp *et al*., 2009).

Artemisinin combination therapy, (ACT) is the most recent combinations therapies for which resistance has been confirmed among *P. falciparum* variants, (Amaratunga *et al*., 2012). Although other conventional therapy like chloroquine has remained the most frequently used drug for *P. falciparum* malaria for many years in the developing world, its status is no longer viable in the treatment of malaria due to drug resistance resurgence, (Malik

et al., 2006). Resistance-conferring alleles for other drugs in Africa have weakened the clinical efficacy of ACTs against malaria parasites and are considered a global health concern that warrants the drive to research fornew anti-malarial drugs across the globe, (Mita *et al.,* 2011). The effort to halt drug resistance is hampered by self diagnosis and use of counterfeit drugs in rural endemic areas of Kenya, (KMIS, 2010).

Herbal remedies are cheap alternatives to conventional medicine have contributed significantly to management of malaria cases. The World Health Organization estimates that 80% of the world's population depends on medicinal plants for their primary health care, (Gurib-Fakim and Schmelzer, 2007; Mothana *et al.*, 2008). The use of traditional medicine has been explored globally and is in use as folklore medicine among people of developing countries in their health care system as an alternative where conventional medicine is less available, (Gupta *et al*., 2010; Rates *et al*, 2001).

Natural products are important sources of new anti-microbial agents which are in the form of secondary metabolites, (Vigneron *et al*., 2005). Drugs derived from unmodified natural products or semi-synthetic drugs obtained from natural sources account for 78 % of the new drugs approved by the United States Food and Drug Administration (FDA) between 1983 and 1994, (Suffredini *et al*., 2006). This evidence contributes to the support and quantification of the importance of screening natural products. The bio-activities of plants have been investigated by a number of researchers worldwide giving very positive and promising results, (Hannan, 2011). Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity, (Parekh and Chanda, 2007). Scientists interest in medicinal plants has burgeoned in recent times due to increased efficiency of new plants derived drugs and rising concerns about the side effects of modern medicinal substances, hence the need to look for new molecular structures as lead compounds from the plant kingdom, (Mariita *et al*., 2010). The bio-activity of natural products is mainly associated with secondary metabolites, often elaborated for the plant's defense. Some of these phytochemicals accidentally protect humans against pathogens. Phytochemicals are known to have several properties important to cells including; prophylactic properties, therapeutic properties, provide nutrition for normal cell health and repairs, inhibit carcinogens and act as antioxidants, (Ngoci *et al*., 2011). The phytochemicals screening of plant materials to determine the presence of bio-active chemical constituents is thus vital in the knowledge of their therapeutic properties, (Awoyinka *et al*., 2007). Despite the beneficial effects of phytochemicals, studies have established that they can be toxic, (Orech *et al*., 2005). These

phytochemicals are produced as part of the plant's defense against pests and herbivores or to gain an advantage over competing agents. Therefore, medicinal plants are not always safe, (Orech *et al*., 2005). It is therefore appropriate to evaluate the potential toxicity of the plant extracts said to indicate useful anti-malarial activity. This will be used to create awareness of the safety of the plant extract(s) as an alternative medication or it be harnessed for its potential as anti-malarial leads, (Kareru *et al*., 2007).

Several plants species are used for medicinal purposes and by local communities. These include *Carissa edulis*, which is locally known in Luo community as "ochuoga"; *Azadirachta indica,* locally known as "mwarubanne / dwele"; *Cassia siamea*, locally known as "ndege", and *Harrisonia abyssinica*, locally known as "pedo", are widely used for their perceived medicinal value in treating various ailments (e.g. malaria, diabetes, eczema, leprosy, fever, dysentery, gastro-intestinal, chest pain, haemorrhoids and snakebite). The phytochemical screening of the four plant species has not been adequately addressed to determine their bioactive phytochemical constituents' therapeutic properties, (Awoyinka *et al*., 2007). Though most of the phytochemicals found in these medicinal plants are known (alkaloids, tannins, saponins, steroids, terpenoids and flavonoids), (Gjorgieva *et al*., 2011; Balammal *et al*., 2012) their anti-malarial activities have not been widely studied There has been significant increase in the use of medicinal plants due to their minimal side effects, availability and acceptability to the majority of the people, (Chhetrict *et al*., 2010).

1.2 Statement of the problem

Falciparum- malaria is the leading cause of human morbidity and mortality, among children below five years old worldwide. This is partly attributed to emergence of strains that are resistant to the available anti-malarial drugs. This has rendered the current drugs as either less effective or completely ineffective in the treatment and management of *falciparum*-malaria cases There is therefore an urgent need to search for new or alternative therapeutic agents. This study investigated in vitro antiplasmodial activities from four medicinal plants that could be used in the control of *Plasmodium falciparum* strains.

1.3 Objectives of the study

1.3.1 General objective

Determination of antiplasmodial activities of *Carissa edulis, Azadirachta indica, Cassia siamea* and *Harrisonia abyssinica* on *Plasmodium falciparum* from Masumbi village, Siaya County, Kenya.

1.3.2 Specific objectives

1. To determine the phytochemicals of methanolic extracts of Carissa *edulis*, *Azadirachta indica*, *Cassia siamea*, and *Harrisonia abyssinica* from Masumbi village, Siaya County, Kenya.

2. To compare the antiplasmodial effect of methanolic and aqueous extracts of *Carissa edulis, Azadirachta indica, Cassia siamea*, and *Harrisonia abyssinica* on chloroquine resistant and chloroquine sensitive, and mefloquine resistant and mefloquine sensitive *Plasmodium falciparum* strain

3. To determine the cytotoxicity of methanolic extracts of *Carissa edulis*, *Azadirachta indica*, *Cassia siamea*, and *Harrisonia abyssinica* on brine shrimp larvae (*Artemia salina nauplii*).

1.4 Justification of the study

Plasmodium falciparum has developed resistance to most current available anti-malarial drugs (e.g. sulfadoxine pyrimethamine and chloroquine diphosphate). *Plasmodium falciparum* develop resistant to anti-malarial drugs due to mutant strains, which may happen as a result of exposure to drugs considered to be under dose. This has elicited need for newer ones to replenish those lost to resistance. Although antimalarial combination studies are underway, information for *in vitro* activities of methanolic and aqueous extracts have not been documented.

Carissa edulis, *Azadirachta indica. Cassia siamea* and *Harrisonia abyssinica* serve as alternative medicinal drugs for the management of *falciparum* malaria because of their availability in the area. Siaya County, Kenya is one of the endemic areas for *Plasmodium falciparum*. Continuous research for new antimalarial molecules is an important aspect to coop with the resistance trend of the parasite. The study finding could be used to reduce the rising rate of resistance to antimalarial drugs by *Plasmodium falciparum*. The study findings could be used as a national guide on malaria. Information is therefore required to establish the potency of the four medicinal plants as alternative medicinal drugs for the management of malaria. This study was undertaken to determine the antiplasmodial activities of the four medicinal plants (*Carissa edulis*, *Azadirachta indica*, *Cassia siamea* and *Harrisonia abyssinica*) against *Plasmodium falciparum* strains.

CHAPTER TWO
LITERATURE REVIEW

2.1 Human malaria

Malaria disease is one of the major public health problems worldwide despite advances in knowledge in malaria. It continues to cause significant morbidity and mortality worldwide due to *Plasmodium falciparum* development of resistance to the available anti-malarial compounds. Previous studies have shown that, it causes over one million deaths each year. World Health Organization (WHO) estimates about 300 - 500 million people being infected with malaria every year, (Kinfu *et al*., 2012).

Although the disease is usually widespread in hot humid regions of Africa, Asia, South and Central America, over 90 % of all malaria cases are found in sub - Saharan Africa. This may be due to *Plasmodium falciparum* resistance to available antimalarial drugs. Apart from *Plasmodium falciparum* resistance to antimalarial compounds, stringent poverty, poor access of health care facilities, lack of knowledge and ignorance about the disease may facilitate the development of *Plasmodium falciparum* resistance to drug. The sub-tropical and tropical region of Africa favours the transmission of the disease throughout the year because of climatic condition that favours the survival of *Anopheles* mosquito that transmits the *Plasmodium falciparum.* Among the five species of *Plasmodium* that cause malaria, almost 85 % of malaria cases are caused by *Plasmodium falciparum* which has been found to be responsible for about 90 % of the deaths in pregnancy women, (Kane *et al*., 2011).

Malaria has been confirmed to be one of the leading causes of morbidity and mortality in Kenya. Malaria - related morbidity and mortality has been in the increasing primarily as a result of increased resistance caused by *Plasmodium falciparum* to the common first line drugs - chloroquine (CQ) and sulphadoxine pyrimethamine, (Shujatullah *et al*., 2012). World Health Organization has recommended treatment policies for *falciparum* malaria in all countries experiencing *Plasmodium falciparum* resistance to monotherapy. In Siaya county resistance of this drug combination has been cited to be facilitated due to use of counterfeit antimalarial drugs, (KMSI, 2010). Widespread resistance caused by *Plasmodium falciparum* to commonly available antimalarial drugs has necessitated countries to review and deploy new anti-malarial drug policies to ensure effective management. Scientists globally are busy trying to understand the phenomenon of *Plasmodium falciparum* developing resistance to antimalarial drugs by understanding the biology and the life cycle. The parasite stages both in the vector and human helps in the understanding on how to control the parasite by monitoring

the mode of drug action administered in crude form orally or in purified forms as injectable (Rusk *et al.*, 2013).

The five commonly known species of *Plasmodium* that infect human beings are *Plasmodium malariae, Plasmodium vivax, Plasmodium ovale, Plasmodium knowlesi* and *Plasmodium falciparum* of which they share common features, (Amaratunga *et al*., 2012).

2.1.1 Stages of *Plasmodium* species

The malaria parasite stages e.g. *sporozoite, trophozoite, schizont, merozoite* and *gametocyte* involves two hosts. During a blood meal, malaria - infected female *Anopheles* mosquito inoculates *sporozoites* into the human host. *Sporozoites* infect liver cells of the human host and mature into *schizonts*, which rupture and release *merozoites* of *Plasmodium falciparum*. After this initial replication in the liver (exo - erythrocytic *schizogony*), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic *schizogony*) of human host. *Merozoites* infect red blood cells. The ring stage *trophozoites* mature into *schizonts,* which rupture and releases *merozoites.* Some parasites differentiate into sexual erythrocytic stages called *gametocytes*. Blood stage parasites are responsible for the clinical manifestations of the disease. Figure 1 below explains the three life cycles of the *Plasmodium falciparum* taking place both in human and in the *Anopheles* mosquito, (Global Health, 2012).

2.1.2 Sporozoites stage of *Plasmodium falciparum*

Plasmodium sporozoites have enabled a gene-based approach to studying this important stage, (Douglas *et al*., 2012). Sporozoites developing within the hepatocyte undergo a radical transformation within the first few hours post invasion. The intracellular sporozoite within its PV settles near the nucleus of host cells and modifies its long and polarized shape to become spherical, (Stephanie *et al*.,2010) The liver stage parasite grows and replicates within the PV ultimately releasing membrane bound bundles of thousands of daughter merozoites, (merosomes) that then enter the erythrocytic cycle, (Balbir *et al*., 2010). Pre-erythrocytic stages are considered targets for the development of vaccines and prophylactic drugs, (Solomon *et al*., 2009).

Liver stage drugs have the potential to hit new targets that are not present or essential during the blood stages of malaria, and these drugs would enjoy a tactical advantage over blood stage drugs because many fewer parasites are involved, which should delay the development of resistance . Liver stage drugs may also have activity against the hypnozoites

of *P. vivax*, like primaquine, and thereby prevent relapsing malaria. Despite their apparent advantages, progress in developing liver stage drugs has been very limited, and this lack of success undoubtedly reflects the way drugs are currently discovered: large numbers of molecules are screened for blood stage activity and only selected active candidates are screened in the liver stage Primaquine, anti-folates, and atovaquone were drugs with liver stage activity, but among these only primaquine had shown anti-hypnozoites activity, (Philippa *et al*., 2005)

Primaquine, a distant chemical relative of chloroquine whose target was yet to be determined, could inhibit both liver stage infections and blood stage parasites *in vitro* (Anaïs *et al*., 2008) and also prevent relapsing malaria, (Alfred *et al*., 2007). Unfortunately, its tendency to cause hemolytic anemia in patients with glucose-6-phosphate dehydrogenase deficiency, the most common human enzyme deficiency, had severely restricted its use. This deficiency was most common in certain parts of Africa, (Annan *et al*., 2006), thus eliminating any hope of using primaquine in an eradication campaign. However, while primaquine itself could never be a widely used anti-malarial agent, its existence argued that other molecules with better therapeutic potential probably existed, (Philippa *et al*., 2005).

Additional evidence for the likelihood of such drugs came from transcriptional profiling of blood and liver stage parasites. Several reports had analyzed both transcriptome and proteome expression levels of malaria parasites in different life stages.

2.1.3 Blood stages (trophozoites, schizonts and merozoites)

The clinical manifestations of malaria stem from replication of asexual blood stages of *Plasmodium* spp. in circulating erythrocytes. In the case of the most dangerous malarial species, *Plasmodium falciparum*, the parasite develops intracellular over the course of about 48 hours to produce a mature schizont containing around 16 daughter merozoites. The schizont eventually ruptures, releasing the merozoites which rapidly invade fresh erythrocytes, thus perpetuating the cycle. Erythrocyte invasion comprises several discrete steps, (Shailja *et al*., 2010), and is facilitated by the discharge of adhesive ligands and other proteins onto the parasite surface from specialized apical organelles called rhoptries and micronemes. Invasion is also accompanied by efficient proteolytic shedding of certain parasite surface and micronemal proteins. These include a micronemal type I integral membrane protein called AMA1 and an abundant glycosyl phosphatidylinositol-anchored merozoite surface protein called MSP1 (which forms a complex with two additional proteins called MSP6 and MSP7, collectively referred to as the MSP1/6/7 complex). Shedding of

these proteins goes virtually to completion upon invasion, (Moritz *et al*., 2009). Previous studies have demonstrated that shedding of *P. falciparum* AMA1 (PfAMA1) and the MSP1/6/7 complex, as well as another micronemal protein called PTRAMP, is all mediated by the same membrane-associated, calcium-dependent parasite subtilizing-like serine protease called PfSUB2, which is itself released from micronemes onto the merozoite surface during invasion, (Rosanna *et al*., 2006).

Malaria arises from infection of erythrocytes by single-cell parasites belonging to the genus *Plasmodium*, the species *P. falciparum* causing the most severe forms of the disease. The formation of a moving junction (MJ) between the membranes of the parasite and its host cell is essential for invasion. Two important components of the MJ are Apical Membrane Antigen 1 (AMA1) on the parasite surface and the *Plasmodium* rhoptry neck (RON) protein complex that is translocated to the erythrocyte membrane during invasion. The extra-cellular region of RON2, a component of this complex, interacts with AMA1, providing a bridge between the parasite and its host cell that is crucial for successful invasion. The parasite thus provides its own receptor for AMA1 and accordingly this critical interaction is not subject to evasive adaptations by the host. Figure 1 below explains the three life cycles of the *Plasmodium falciparum* taking place both in human and in the *Anopheles* mosquito, (Global Health, 2012).

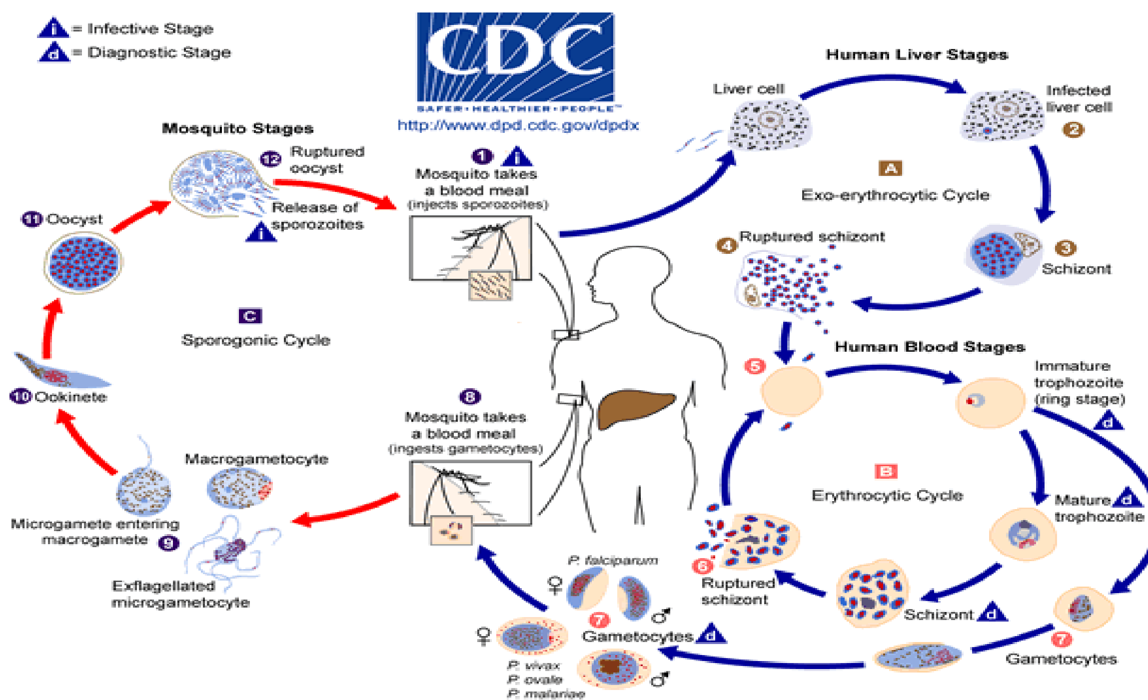

Figure 1: *Plasmodium* life-cycle (*http://www.dpd.cdc.gov/dpdx*)

During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates 1. *sporozoites* into the human host as illustrated by 2. *Sporozoites* infect liver cells as illustrated by and mature into 3. *Schizonts* rupture and release 4.*merozoites*. After this initial replication in the liver A, (exo - erythrocytic *schizogony*), the parasites undergo asexual multiplication in the erythrocytes B, (erythrocytic *schizogony*). *5, Merozoites* infect red blood cells. 6, the ring stage *trophozoites* mature into *schizonts*, and some parasites differentiate into sexual erythrocytic stages 7(*gametocytes*). Therefore blood stage parasites are responsible for the clinical manifestations of the disease 8., The *gametocytes*, male (*microgametocytes*) and female *(macrogametocytes*) are ingested by an *Anopheles* mosquito during a blood meal and 9, while in the mosquito's stomach; the micro *gametes* penetrate the *macrogamete* generating *zygotes*. 10, the *zygotes* in turn become motile and elongated (*ookinetes*) which invade the midgut wall of the mosquito where they develop into 11, *oocysts* 12. The *oocysts* grow, rupture, and release *sporozoites* which make their way to the mosquito's salivary glands. Inoculation of the *sporozoites* into a new human host perpetuates the malaria life cycle once again (*http://www.dpd.cdc.gov/dpdx*).

2.2 Anti - plasmodial drugs (quinine and antifolates)

The numbers of drugs that can be used to treat or prevent malaria are limited. The most widely used are quinine and its derivatives and antifolates combination drugs (Gesase *et al*., 2009).

2.2.1 Quinine and related compounds (chloroquine, primaquine and mefloquine)

Quinine, along with its dextroisomer guanidine, has been the drug of last resort for the treatment of malaria which is severe and complicated disease. Chloroquine is a 4 - aminoquinoline derivative of quinine first synthesized in 1934 and has since been the most widely used antimalarial drug. Historically, it has been the drug of choice for the treatment of non-severe or uncomplicated malaria and for chemoprophylaxis. Drug resistance has dramatically reduced its usefulness. Amodiaquine is a relatively widely available compound closely related to chloroquine. Other quinine - related compounds in common use include primaquine, and mefloquine, (Gesase *et al*., 2009).

2.2.2 Antifolates combination drugs (proquanil, chloroproquanil, pyrimethamine and trimethoprim)

Antifolates combination drugs are various combinations of dihydrofolate - reductase inhibitors (e.g. proguanil, chloroproquanil, pyrimethamine, and trimethoprim) and sulfa drugs

(e.g. dapsone, sulfalene, sulfamethoxazole, sulfadoxine, and others). Although they have antimalarial activity when used alone, parasitological resistance can develop rapidly. When used in combination, they produce a synergistic effect on the parasite and they are effective even in the presence of resistance to the individual components. Typical combinations include sulfadoxine / pyrimethamine, sulfalene pyrimethamine and sulfamethoxazole - trimethoprim. A new antifolate combination drug was being tested in Africa. This drug, a combination of chloroproguanil and dapsone, also known as Lap - Dap, was found to have much more potent synergistic effect on malaria than existing drugs such as Sulphadoxine pyrimethamine. Benefits of this combination included 1) a greater cure rate, even in areas that were experiencing some level of sulfadoxine pyrimethamine resistance. 2) A lower likelihood of resistance developing because of a more advantageous pharmacokinetic and pharmaco - dynamic profile, and 3) probable low cost, (Smith *et al*., 2011).

2.2.3 Antibiotics compounds (tetracycline and derivatives such as doxycycline)

Tetracycline and derivatives such as doxycycline are very potent antimalarial and are used for both treatment and prophylaxis. In areas where response to quinine has deteriorated, tetracycline was often used in combination with quinine to improve cure rates. Clindamycin has been used but was realized to offer only limited advantage when compared to other available antimalarial drugs. Parasitological response was slow to clindamycin and recrudescence rates were high, (WHO, 2006).

2.2.4 Artemisinin compounds (artesunate and artemether)

A number of sesquiterpine lactones compounds have been synthesized from the plant *Artemisia annua* including (artesunate, artemether). These compounds have been used for treatment of severe malaria and have shown very rapid parasite clearance times and faster fever resolution than occurs with quinine. In some areas of South - East Asia, combinations of artemisinin and mefloquine have offered the only reliable treatment for even uncomplicated malaria, due to the development and prevalence of multidrug resistant *falciparum* malaria (Nosten and White, 2007). Combination therapy (artemisinin compound given in combination with another antimalarial, typically a long half - life drug like mefloquine) has reportedly been responsible for inhibiting intensification of drug resistance and for decreased malaria transmission levels in South - East Asia, (Price *et al*., 1996).

World Health Organization (WHO) has recommended artemisinin - based combination therapies (ACTs) for the treatment of uncomplicated malaria caused by the *Plasmodium falciparum* parasite. Combining two active ingredients with different mechanisms of action, ACTs are the most effective antimalarial medicines used for treatment of malaria. The choice of ACTs has been based on the results of therapeutic efficacy studies against local strains of *Plasmodium falciparum* malaria. ACTs are therefore the mainstay of recommended treatment for *Plasmodium falciparum* malaria. World Health Organization recommends that national malaria control programmes regularly monitor the efficacy of antimalarial medicines in use, (WHO, 2012).

World Health Organization recommended that, Artemisinin and its derivatives must not be used as oral monotherapy, as this promotes the development of parasite resistance to artemisinin. Instead fixed - dose formulations (combining two different active ingredients co-formulated in one tablet) are strongly preferred and recommended. Co - blistered, co - packaged or loose tablet combinations, have been found not to facilitate adherence to treatment and reduce the potential use of the individual components of co - blistered medicines as monotherapy (WHO, 2010).

2.2.5 Miscellaneous compounds (halofantrine and atovaquone)

Halofantrine is a phenanthrene - methanol compound with activity against the erythrocytic stages of the malaria parasite. Its use has been especially recommended in areas with multiple drug-resistant *falciparum,* (Asthana *et al*., 2001). Atovaquone is a hydroxynapthoquinone that was used most widely for the treatment of opportunistic infections in immunosuppressed patients. It was effective against chloroquine - resistant *P. falciparum*, but because, when used alone, resistance develops rapidly, atovaquone was usually given in combination with proguanil, (Looareesuwan *et al*., 1996). A new fixed dose antimalarial combination of 250 mg atovaquone and 100 mg proguanil (Malarone) was brought to market worldwide and additionally distributed through a donation programme, (Guthmann *et al*., 2005). The two drugs originally synthesized in China and underwent field trials. Pyronaridine was reportedly 100 % effective in one trial in Cameroon, (WHO, 2006); however, it was only between 63 % and 88 % effective in Thailand. Lumefantrine, a fluoromethanol compound, was instead produced as a fixed combination tablet with artemether, (Nkhoma *et al*., 2007).

2.2.6 Combination antimalarial therapy as two antimalarial drugs

The use of two antimalarial simultaneously, especially when the antimalarial have different mechanisms of action, has the potential for inhibiting the development of resistance to either of the components. The efficacy of a combination of a 4 - aminoquinoline drug (either chloroquine or amodiaquine) with sulfadoxine / pyrimethamine was reviewed. The review suggested that the addition of either chloroquine or amodiaquine to sulfadoxine / pyrimethamine marginally improved parasitological clearance (compared with sulfadoxine / pyrithemine alone). The studies reviewed were mostly done in areas and at times when sulfadoxine / pyrithemine and chloroquine / amodiaquine retained a fair amount of efficacy, and it was not clear from these studies how well such a combination would act in areas where one of the components was significantly compromised. Additionally, there were no data to suggest whether this slightly improved clearance would translate into prolonged useful life span for either drug, (Guthmann *et al*., 2005).

2.3 Status of drug - resistant malaria

Resistance to antimalarial drugs has been described for two of the five species of malaria parasite that naturally infect humans, *Plasmodium falciparum* and *Plasmodium vivax*. *Plasmodium falciparum* has developed resistance to nearly all antimalarial in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly. *P. vivax* infections acquired in some areas were showed to be resistant to chloroquine and /or primaquine, (Dondorp *et al*., 2009). Chloroquine - resistant *P. falciparum* malaria has been described everywhere that *P. falciparum* malaria has been transmitted except for malarious areas of Central America (north - west of the Panama Canal), the island of Hispaniola, and limited areas of the Middle East and Central Asia. Sulfadoxine pyrimethamine resistance occurs frequently in South - East Asia and South America. Sulfadoxine pyrimethamine resistance became more prevalent in Africa as that drug was increasingly being relied upon as a replacement for chloroquine. Mefloquine resistance was frequent in some areas of South - East Asia and was reported in the Amazon region of South America and sporadically in Africa, (Nkhoma *et al*., 2007). Cross - resistance between halofantrine and mefloquine was suggested by reduced response to halofantrine when used to treat mefloquine failures, (Ngemenya *et al*., 2006).

Apart from conventional drugs medicinal plants have taken role as alternative medicine in the management of malaria. The need to development improved therapeutics for treatment of parasitic diseases is pressing, and particularly for the treatment of malaria. Drug

resistance developed by *Plasmodium falciparum* to cheap and affordable antimalarial drugs such as chloroquine and sulfadoxine / pyrimethamine was widespread. More so, the gradual decline in the efficacy of artemisinin - based combination therapies (ACTs) in some malaria endemic areas is a cause for concern, (Dondorp, *et al*.,2009). At present there is no effective vaccine and resistance of the vector to insecticides being a problem. Therefore, there is need for the development of novel entities that would be effective against resistant parasite, especially *Plasmodium falciparum,* (Ridley, 2002).

2.4 Medicinal plants with antimalarial activity

A number of plant species contain biologically active ingredients that are used for the treatment of various infectious diseases, (Nisar *et al*., 2011; Premkumar *et al.*, 2011). Phytochemicals have some physiological effect, and their medicinal values are due to substances found in tissues that produce a definite physiological action on the human body, (Tyrupathi *et al*., 2011). Species of trees have been identified that contain chemicals displaying medicinal or therapeutic properties, (Kumar *et al*., 2009). The most important of these substances are the alkaloids, tannins, saponins, steroids, terpenoids and flavonoids, (Gjorgieva *et al*., 2011). There has been significant increase in the use of medicinal plants due to their minimal side effects, availability and acceptability to the majority of the people. Therefore medicinal plants play an important and vital role in traditional medicine and are widely consumed as home remedies, (Balammal *et al*., 2012). Different parts such as (roots, leaves, bark and flowers) of medicinal plants can be extracted with boiling water or decoction process (Chhetrict et *al*., 2010). The following are medicinal plants; (Carissa *edulis*, *Azadirachta indica, Cassia siamea* and *Harrisonia abyssinica*) are used for the management of malaria worldwide.

2.4.1 Description of *Carissa edulis*

Carissa edulis is thorny and evergreen shrub, usually 5 meters or more. Has a grey bark, smooth with straight woody spines of 5 centimeters (cm) long often in pairs, and rarely branched. Has milky latex in all families. Leaves are opposite, leathery, dark green, shiny to 5cm, with pointed tip, rounded base with very short stalk. Flowers are fragrant, pink – white terminal clusters with each flower to 2cm, lobes overlap to the right. Fruit are rounded berries and approximately 1cm, purple - black when ripe, sweet and edible with 2 - 4 seeds per fruit, (Rukunga, 2008). *Carissa edulis* is widespread in Africa from Senegal to Somalia and South, Africa to Botswana and Mozambique, and in Asia; from Yemen to India. Common in most

areas of Kenya in forest edges, and thickets, hillsides, rock places, black cotton soils, valley bottoms, seasonally flooded areas, and rare in coastal belt where rainfall range between 500 - 1800mm, (Rukunga, 2008). Classification of *C.edulis*, see Table 1.

Table 1: Classification of *Carissa edulis*

Kingdom	Plantae
Phylum	*Trachaophyta*
Class	*Magnoliopsida*
Order	*Gentianales*
Family	*Apocynaceae*
Scientific name	*Carrisa edulis*
Species	*edulis*

2.4.2 *Carissa edulis* as a medicinal plant

In Kisii, decoction of *Carissa edulis* was established to treat malaria by a study done on physical evaluation of selected eight medicine plant used for the treatment of diabetes, malaria, and pneumonia, (Maobe *et al*., 2013). The study was carried to determine the pH levels of the aqueous extracts of selected herbs used for the treatment of the malaria. The study used crude extract without further partition of the solvent that could result in loss of synergism of the compounds, (Kebenei *et al*., 2011). A study carried out on an inventory of medicinal plants that the people of Nandi use to treat malaria was done to address the use of medicinal plants for treatment or prevention of human ailments by Nandi people. However the issue of drug resistance caused by *Plasmodium falciparum* was not addressed, (Jeruto *et al*., 2011).

A study done on antiplasmodial activity and toxicity of extracts of plants used in traditional malaria therapy in Meru and Kilifi Districts of Kenya showed no antiplasmodial activities in *Carissa edulis,* (Kirira *et al*., 2006).This may prove the ecological diversity of *Carissa edulis* and different phytochemical that may be found at different locations due to climatic differences and spatial distribution. *Carissa bispinosa* is occasionally found at the coast and shares many features with *Carissa edulis. Carissa tetramera* is restricted at the coast. Astudy carried out on Preliminary phytochemical and pharmacognostical evaluation of *Carissa edulis* leaves was done to establish the morphology and microscopic characteristic

*(*Neeli *et al*., 2013). However The *in vitro* antimalarial efficacy of *Carissa edulis* as medicinal plants is requierd (Rukunga *et al*., 2008).

2.4.3 *Azadirachta indica*

This is the fast growing evergreen popular tree. It is found commonly in India, Africa and America. It has been used in ayurvedic medicine for more than 4000 years due to its medicinal properties, (Pingale *et al*., 2010). It is a 'village dispensory' in India. Its importance has been recognized by the United State National Academy of Sciences which published its report in 1992, (Biswas, 2002). *Azadirachta indica* A. Juss is found in Southeast Asia and West Africa; the plants were planted in the Caribbean and several Central American Countries, including Mexico, (Sergio *et al* 2007).

Azadirachta indica A. Juss has been used as the first line of treatment for 60 % of Nigerian children with malaria associated fever, (WHO, 2003). It has also been reported to be useful in several areas in many countries. Apart from the treatment of malaria, diabetes, worms, cardiovascular and skin diseases, *Azadirachta indica* A.Juss has been used as an insecticide, insect repellant and oral dentifrice, (Akaneme *et al*., 2012).

It has maximum use for non wood products such as leaves bark, flowers, fruits, seeds and gum oil than any other tree species. It is known to have anti allergenic antidermatic, antifedent, antifungal and other biological activities. Due to these activities *Azadirachta indica* A. Juss has become important in the global context because it offers answers to major problems facing mankind, (Phayanithi *et al.,* 2010).

Azadirachta is a member of Mahogany family and the species of *Azadirachta* that has been reported are *Azadirachta indica* A. Juss - native to Indian subcontinent and *Azadirachta excels Kack* - confined to Philippines and Indonesia. Classification of *Azadirachta indica*, see Table 2.

Table 2: Classification of *Azadirachta indica*

Kingdom	*Plantae*
Subkingdom	*Viridaeplantae*
Division	*Trachaophyta*
Subdivion	*Spermatophytina*
Class	*Magnoliopsida*
Order	*Sapindales*
Family	*Meliaceae*
Genus	*Azadirachta* A. Juss
Species	*indica*

2.4.4 *Cassia siamea*.

Cassia species (*Caesalpinaceae*) are well known medicinal plant commonly found in India and other tropical countries. The species is found in Kenya and various medicinal properties have been attributed to this plant in the traditional system of Indian medicine. Several parts of *Cassia siamea* Lam have been used for the treatment of malaria, a tropical endemic disease with high morbidity and mortality. The leaves have been found to be the most useful parts, because it is frequently used especially in Africa and Asia continents. Decoction of leaves has been used for body bath to treat malaria and liver disorders. Although in Cote d'Ivoire, the decoction of leaves is administered orally for treatment of cough, stomach pains and malaria. *Cassia siamea* Lam stems have been used for orally as treatment well as body bath against malaria and liver disorders in Burkina Faso, Ghana and Nigeria, (Mamaden *et al*., 2014).

Several anthraquinone have been isolated from the seeds of *Cassia* species. Sennosides, which are well known for their medicinal importance, have been detected in the leaves of the plant. *Cassia* species are already reported in the ancient ayurvedic literatures and literature survey indicated its use against various skin diseases such as ringworm, eczema, and scabies. Because of the high incidence of skin diseases, especially among the poor community of the Indian population, it was felt worthwhile undertaking research on this plant. According to ayurvedic, the leaves and seeds are acrid, laxative, anti-periodic, anti-helmintic, ophthalmic, liver tonic, cardio tonic and expectorant. The leaves and seeds are

useful in treatment of leprosy, and ringworm, (Shivjeet *et al*., 2013). Classification of *Cassia siamea,* see Table 3.

Table 3: Classification of *Cassia siamea*.

Kingdom	*Plantae*
Subkingdom	*Tracheobiota*
Superdivision	*Spermatophyta*
Division	*Magnophyta*
Class	*Magnoliopsida*
Sub class	*Rosidae*
Order	*Fabales*
Family	*Fabaceae*
Genus	*SennaMill*
Species	*Siamese cassia*

2.4.5 *Harrisonia abyssinica*.

This is an evergreen shrub or tree and sometimes climbing. It is commonly found growing in riverside, vegetation; in dry bush land, wooden grasslands, and the costal forest margins at altitude coastal levels to 1600 meters. Medicinal applications of the plant include being used as treatment for a number of diseased conditions such as veneral diseases, fever, malaria, diarrhea, urinary problems and intestinal worms. Leaf sap is drunk for treatment in general body pains. Root and the bark decoctions have been used to treat malaria, abdominal pain, hemorrhoids and snake bite. Root bark of *Harrisonia abyssinica* Oliv., is been known to exhibit antiplasmodial activities, (Adhikari *et al*., 2007).The genus *Harrisonia* comprises of 3 species, 2 of which occur in tropical Asia. *Harrisonia abyssinica* Oliv. is widely distributed in most tropical regions of Africa. *H. abyssinica* is variable, especially in size, shape and hairiness of the leaves. The methanolic and water extracts from the stem bark have shown moderate antiplasmodial activity in some *in-vitro* study, (Balde *et al*., 2001). Classification of *Harrisonia abyssinica*, see Table 4.

Table 4: Classification of *Harrisonia abyssinica.*

Kingdom	*Plantae*
Unranked	*Angiosperms*
unranked	*Endicots*
Unranked	*Rosids*
Order	*Sapindales*
Family	*Rutaceae*
Genus	*Harrisonia*
Species	*abyssinica*

CHARPTER THREE

MATERIALS AND METHODS

3.1 Description of study area

Siaya county falls within latitudes 0° 26´ North to 0° 90´ South and longitudes 33° 58´ East and 34° 35´ West. It is traversed by the equator, thus falling within inter tropical convergence zone (ITCZ). The county falls within the Lake Victoria Drainage Basin. It has lakes, swamps, permanent and seasonal rivers and streams. Apart from bordering the second largest fresh water lake in the world, Siaya County hosts the largest wetland in Kenya, the Yala swamp. This wetland covers 17,500 hectares and is a Trans - county resource between Siaya and Busia counties. It also hosts Lake Kanyaboli, one of the country's rare Ox bow lakes with an average dept of 3 meters and a total area of 1500 hectares.

Since the county is located in the Inter Tropical Convergence Zone it experiences a modified equatorial climate that is influenced by relief and numerous hydrological bodies. It has a mean rainfall ranges between 800 - 1600 mm with a mean temperature of about 22.5 ^{o}C. There are two rainy reasons, long rains from March to May and short rains from October to November. The county is wetter on the high altitude of the eastern side and dry to the western part towards Bondo. Masumbi village in Siaya county lies on the eastern side that experiences wet climatic conditions. This favours the longetivity and the survival of the *Anopheles* mosquitoes that transmits the *P. falciparum* throughout the year, see Figure 2.

Figure 2: Map of Kenya and Siaya county (*www.kenyampya.com/index.php?County*)

Key: ** is sampling sites in Masumbi village in South East Alego, Siaya County (http//www.opendata.go.ke/Facet/counties/Siaya)

3.2 Objective 1: Determination of phytochemicals of methanolic extracts of *Carissa edulis, Azadirachta indica, Cassia siamea* and *Harrisonia abyssinica*

3.2.1 Collection of plant samples

The plants' root barks stem barks, and leaves for the study were collected from Masumbi village, Luo community, Siaya county, Kenya from December 2014 - January, 2015. The plants were identified through ethnobotanical approach by consulting the herbalist from the village and the sample size was guided by the main objectives. Roots of *Carissa edulis* were dug using a hoe and young tap roots collected from several plants of the same species because the Thomas Wiley Laboratory Mill model 4 could not grind hard substances. Young parts of the plants were considered active parts of the plants and were expected to contain concentrated phytochemicals. This was repeated with *Harrisonia abyssinica* Oliv roots. Collection of other parts (leaves, and stem bark) for *Azadirachta indica* A. Juss and *Cassia siamea* Lam were collected by physically pruning the young leaves and slashing of stem bark using a knife respectively. For every plant that was sampled, its leaves, branches and / or fruits were carried for taxonomic identification at the Department of Biological Sciences, Egerton University, Kenya (Appendix 5a, Laboratory procedures).

3.2.2 Identification of plant samples

The plants samples were taxonomically identified by a botanist at the Department of Biological Sciences, Egerton University – Njoro campus as described by, (Beentje, 1994). The voucher numbers; V/ SO / 014 / 01 EU, V/ SO / 014 / 02 EU, V/ SO / 014 / 03 EU and V/ SO / 014 / 04 EU were prepared, assigned to the plant samples and preserved in the Departmental herbarium. Preparation of the plants samples were then carried out (Plate 1 and 2 respectively).

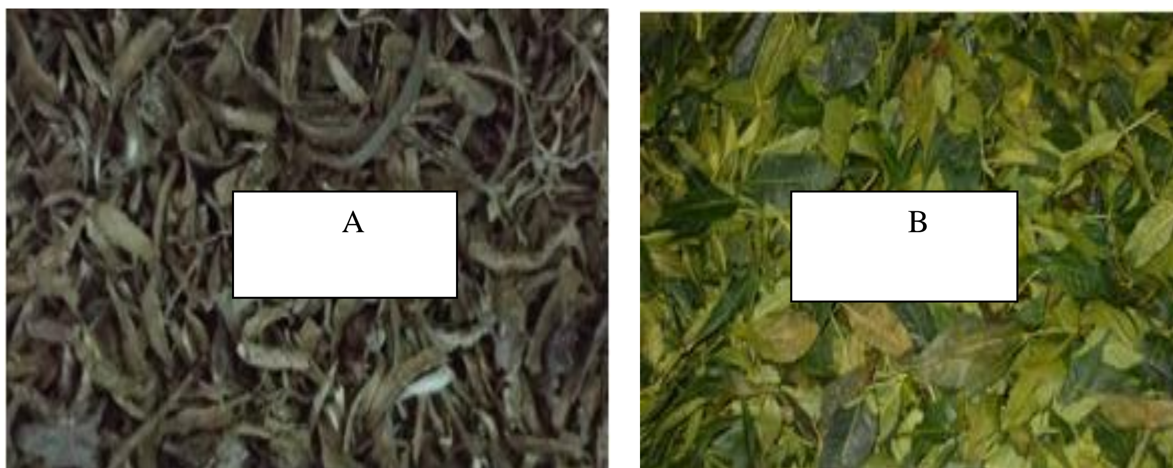

Plate 1: Photographs of A, *Carissa edulis*-root bark; B, *Azadirachta indica* leaves

Plate 2: Photographs of C, *Cassia siamea* -stem back and D, *Harrisonia abyssinica* root bark.

3.2.3 Preparation of plants' root bark, stem bark and leaves

The plants' root bark, stem bark and leave, were prepared by cleaning them from undesirable materials. They were cut into small pieces using a knife and air dried in the dark at room temperature for four week to avoid decomposition of light sensitivity bioactive compound. A constant weight was achieved before grinding them into coarse powder by a mechanical mill (Thomas - Wiley Laboratory mill, Model 4), see Table 5.

Table 5: Plant parts used for extraction and concentration

	Part used	Weight before grinding	Weight-after grinding
Sample A	Root bark	555.173 g	341.898 g
Sample B	Leaves	264.479 g	159.019 g
Sample C	Stem bark	737.315 g	198.632 g
Sample D	Root bark	382.314 g	158.084 g

Key: A= *Carissa edulis*, B= *Azadirachta indica*, C= *Cassia siamea* and D= *Harrisonia abyssinica*.

3.2.4 Extraction of methanolic phytochemicals from plant powdered parts

Approximately 100 g of *Carissa edulis* root bark powder, *Cassia siamea* Lam stem bark powder, *Azadirachta indica* A. Juss leaves powder, and *Harrisonia abyssinica* Oliv root bark powder was macerated into 300 ml of organic solvent (methanol) in 500 ml conical flasks (Pyrex). The preparation was left to stand at room temperature for 72 hours. This was followed by intermittent shaking after every 12 hours. The four different extracts were decanted into 400ml conical flasks. Fresh solvent (100 ml of methanol) was subsequently measured using a 100 ml measuring cylinder, added into each soaked samples and agitated for ten minutes, and further decanted out into 500 ml measuring cylinder each. Filtration was done through, (Whatmann filter paper No.1) by gravity into 1000 ml volumetric flasks after 72 hours for concentration as describe by, (Ngoci *et al*., 2012).

3.2.5 Concentration of methanolic phytochemicals from plants' powdered parts

Preparation of the concentrations was carried out by setting out the rotary vacuum evaporator (BüCHI ROTAVAPOR R-205 V805, Flawil Switzerland). The temperature of the rotary vacuum evaporator was set to 60 °C with 50 revolutions per minute (rpm) and the pressure of 15 mercury (Hg). Each filtrate obtained as a result of filtration was transferred into a 250 ml round bottom flask. The flask was connected to the rotary vacuum evaporator and the suction pump set to allow the process to start. The four volumes of filtrates were concentrated in a rotary vacuum evaporator (BüCHI ROTAVAPOR R-205 V805, Flawil Switzerland) in the Department of Chemistry, Egerton University. The preparations were transferred into 250 ml flat button beakers and covered with perforated alluminium foil. The four were left to air dry in at room temperature in a flow laminar hood for thirty days. The dry weight of each concentrated sample was recorded, kept at 4°C for later use in phytochemical analysis, as described by (Akeng'a, 2005).

3.3 Water extraction and sublimation of plant powdered parts

Another 50 g of each powdered samples of *Carissa edulis* root bark, *Cassia siamea* Lam stem bark, *Azadirachta indica* A Juss leaves and *Harrisonia abyssinica* Oliv root bark were soaked into four different round bottom volumetric flasks each containing 250 ml of water. Each preparation was boiled at 60 °C for 3 hours. The development was left to cool and then decanted into different beakers. The final volumes were filtered using gravitational force through a filter paper (Whatman filter paper No.1). The filtrates were packed in aliquots of 50 ml and taken for further extraction and sublimation process. The process was carried

out in a freeze dryer (4.5 Console Freeze Dryer -220 / 240 V, 50 HZ) for 60 hours at International Centre of Insect Physiology and Ecology (ICIPE), Kenya. The dryer was auto set with a eutectic temperature of -25 ^{o}C to -50 ^{o}C, pre-freeze temperature of -35 ^{o}C to -60 ^{o}C with vacuum set point of 0.22 to 0.01 mBr and lower. The samples were frozen before the actual process of freeze drying began. The water was then removed from the frozen materials by converting the frozen water directly into vapour without formation of liquid water. The vacuum pump was used to enhance the removal of water vapour. The dry weight of aqueous extracts were not interfere with but transported and stored in dry and moisture free cabinet awaiting *in vitro* analysis.

3.4 Screening for unknown compounds from methanolic extracts using thin layer chromatography (TLC)

Although retention factor was not done using known compounds, thin layer chromatography was undertaken as a preliminary test to confirm the presence of unknown compounds in the plant extracts. Thin layer chromatography is a simple, quick, and inexpensive procedure that gives the chemist a quick answer to how many components are in a mixture. Thin layer chromatography is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate). The purpose for running TLC for this research was to establish whether the methanolic extracts had unidentified compounds.

3.4.1 Preparation of thin layer chromatography solvent

Thin layer chromatography (TLC) works on the same principles. In TLC, the stationary phase is a polar absorbent, usually finely ground alumina or l silica particles. The absorbent is coated on a glass slide or plastic sheet creating a thin layer of the particular stationary phase.

Determination of the solvent system to develop a thin layer chromatography (TLC) plate was done in several trials. These tests were run using hexane: dichloromethane, hexane: ethyl acetate, and methanol: dichloromethane. The result was carefully observed and recorded. The ideal solvent system was methanol: dichloromethane that gave the best separation according to this preparation. Thin layer chromatography plates were then made using MeOH 90 ml: DCM 10 ml, MeOH 70 ml: DCM 30 ml and MeOH 50 ml: DCM 50 ml respectively.

3.4.2 Preparation of the developing solvent container

Jars with lids were used as the developing container for the TLC plates. The solvent was poured into the jars to a depth of just less than 0.5 cm. The jars were covered while TLC plates were prepared.

3.4.3 Preparation of the TLC plate

Thin layer chromatography plates used were purchased as 5 cm x 20 cm sheets. Each large sheet was cut horizontally into plates which were 5 cm tall by various widths. The plates were handled carefully not to disturb the coating adsorbent. About 0.5 cm from the bottom of the plate was measured, and using a pencil; a line was drawn across the plate at the 0.5 cm mark. This was the origin line on which the spot was made on the plate. Names of the crude extracts were marked under the line and sample that were placed on the plate with enough space between the samples.

3.4.4 Spotting the TLC plate

About 1 mg of each methanolic crude sample was dissolved in a volume of 1 ml of methanol solution. Micro-caps, measuring 10 µl were used by dipping into the solution and gently touch the end of it onto proper location marked on the TLC plate.

3.4.5 Developing the TLC plate

The Thin Layer Chromatography (TLC) plate was placed in the developing jar, covered and left undisturbed on the bench top. The solvent rose up the TLC plate by capillary action, and care was taken not to allow the solution to cover the spot. The plates were allowed to develop until the solvent was about half a centimeter below the top of the plate. The plates were removed from the jar and immediately the solvent fronts were marked with a pencil. The plates were allowed to dry out.

3.4.6 Visualizing the spots on the TLC plate using UV-light

Separated components of the mixture were visualized by Ultraviolet (UV). The crude methanolic extracts confirmed the presence of unknown compounds because they could eluminate at 254nm with a purple colour which was UV active , see Plate 3.

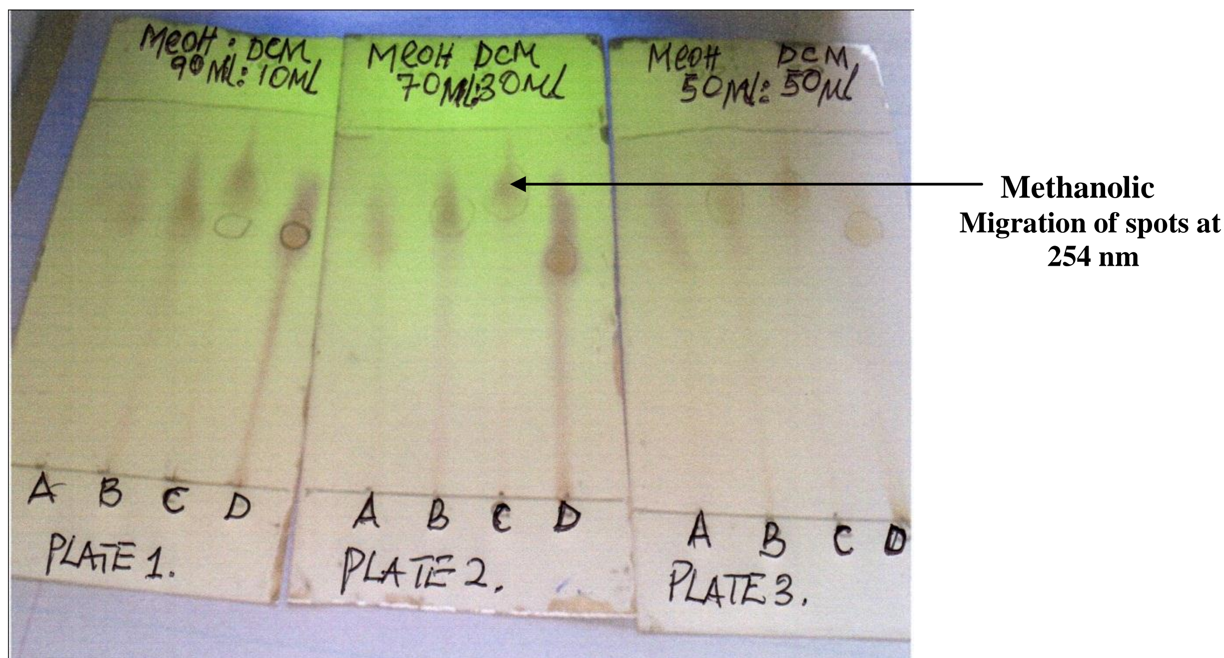

Plate 3: Key A= *Carissa edulis*, B= *Azadirachta indica* A. Juss, C= *Cassia siamea* Lam and D= *Harrisonia abyssinica* Oliv. , MeOH= methanol, DCM= Dichloromethane and (90:10; 70:30; and 50:50) = ratio of solvent systems

3.5 Detecting the presence of phytochemicals in methanolic extracts

The plant extracts were qualitatively tested for the presence of alkaloids, cardiac glycosides, flavonoids, saponins, steroids, tannins and terpenoids, (Oduor *et al*., 2014). Characteristic colour changes identified them. The results were reported as (-) absence of detectable colouration, (+) presence of slight colouration, (++) presence of deep colouration, and (+++) presence of very deep colouration. Each methanolic crude extract was prepared and transferred to test tubes labeled (A for methanolic crude extract of *Carissa edulis*, B for methanolic crude extract of *Azadirachta indica*, C for methanolic crude extract of *Cassia siamea* and D for methanolic crude extract of *Harrisonia abyssinica*).

3.5.1 Test for presence of alkaloids

Each crude methanolic extract was boiled for 15 minutes in 1M HCl (25.0 ml, 1%). Equal volumes of the resulting suspension were filtered into four test tubes (A, B, C and D). To each test tube, 5 drops of freshly prepared Dragendorff's reagent was added, (Akeng'a, 2005).

3.5.2 Test for the presence of cardiac glycosides

About 2 g of each extract was weighed and emulsified in 5ml of water. About 5 ml of each crude methanolic extract was measured using a cylinder (Pyrex) and transferred into different glass test tubes (A, B, C and D). 2 ml of glacial acetic acid containing one drop of ferric chloride solution was measured using a measuring cylinder and added to each methanol crude extract. Avolume of 1 ml of concentrated sulphuric acid (H_2S0_4) was underlayed slowly to each methanol crude extract, (Akeng'a, 2005).

3.5.3 Test for the presence of flavonoids

Approximately 5 ml of dilute aqueous ammonia solution was added to each portion of the aqueous filtrate of crude methanolic extracts in test tubes (A, B, C and D). This was followed by addition of concentrated sulphuric acid (H_2SO_4) to every test tube.

3.5.4 Test for the presence of saponins

About 2 g of each crude methanolic extracts was transferred into glass test-tubes (A, B, C and D). In each test tube, 20 ml of distilled water was added. Each preparation was boiled in a water bath and then filtered. 10 ml of each filtrate was mixed with 5 ml of distilled water and shaken vigorously. 3 drops of olive oil were added to each preparation and then shaken vigorously.

3.5.5 Test for the presence of steroids

About 2 ml of acetic acid and 2 ml of Sulphuric acid ($H_2SO_{4)}$ was added to 0.5 g to each crude methanolic extract in separate glass test tubes (A, B, C and D).

3.5.6 Test for the presence tannins

Approximately 0.5 g of each crude methanolic extract was boiled in 20 ml of distilled water in different glass test-tubes (A, B, C and D). The preparations were filtered, and a few drops of 0.1 % ferric chloride added to each test tube using a dropper.

3.5.7 Test for the presence terpenoids

Approximately 5 ml of each crude methanolic extract was mixed with 2 ml of chloroform ($CHCH_3$) and 3 ml of concentrated sulphuric to form a layer in different glass test tubes (A, B, C and D).

3.6 Objective 2: Determination of antiplasmodial effect of methanolic and aqueous extracts of *Carissa edulis, Azadirachta indica, Cassia siame*a and *Harrisonia abyssinica* on chloroquine - sensitive and mefloquine - sensitive *Plasmodium falciparum* strain.

3.6.1 Activation of *Plasmodium falciparum* strains from liquid nitrogen

Reference *Plasmodium falciparum* in liquid nitrogen was stored for future use. For the revival, they were located in the storage and pulled out for processing (Appendix 5b, Laboratory procedures). They were transferred into bio-safety cabinet and thawed over water bath at 37 °C. The amount thawed was transferred into 15 ml centrifuge tube, and the volume marked. The amount thawed was picked and divided by 5(five). A volume of 12 percent (%) sodium chloride (NaCl) was picked and mixed with the sample. The content was then left to stand at room temperature for 5 minutes. This was followed by 0.2 % and 0.9 % NaCl respectively. About 9 volumes of 1.6 % sodium chloride (NaCl) was added and centrifuged at 1500 rpm for 3 minutes. The supernatant was removed, and 9 volumes of 0.9 % NaCl plus 0.2 % glucose were added and centrifuged at 1500 rpm for 3 minutes to enhance parasite adaptation. The supernatant was removed and the flasks labelled. The pellet was mixed with 4.5 ml of 20 % complete medium with serum (CMS) and 0.5 ml of washed zero positives (0+) red blood cells (RBCs) to homogeneity then transferred to 25 cubic centimeters (cc) culture flasks. Components of the flasks were then gassed with 5 % carbon dioxide (CO_2), 90 % nitrogen (N_2) and 5 % oxygen (O_2) and then incubated at 37 °C under moisture condition, (Amaratunga, *et al*., 2013; Akala *et al*., 2011).

3.6.2 Preparation of *Plasmodium falciparum* culture medium

The *in vitro Plasmodium falciparum* culture was done under sterile condition. A 10.4 g packet of powdered Roswell Park of Memorial Institute (RPMI 1640) medium was weighed and added to the cylinder containing 960 ml of phosphate buffer solution. Another 5.94 g of HEPES was weighed into a clean boat and poured into a cylinder containing 960 ml of phosphate buffer solution. A magnetic stirrer was put in the cylinder, covered it with a clean Parafilm paper, and stirred using the magnetic stirrer until the components were completely mixed and dissolved. The volume of the content was topped up to one litre. The preparation was filtered using 0.2 µm filter unit, and labelled with the expiry date, (Trager and Jensen, 1976).

3.6.3 Maintaining *Plasmodium falciparum* strain in RPMI 1640 culture

Media was changed by removing flasks containing complete medium with serum (CMS) 10 % from the refrigerator and warmed in 37 °C in an incubator for 10 minutes and then placed in a bio-safety cabinet. Caps were subsequently removed from culture flasks. The culture flasks were held at 45 degrees or tilted to allow the media to flow towards the corner to be aspirated until 0.5 ml of medium and red blood cell was left.

Clean and sterile microscopic glass slide were removed from the slide packet and placed on the working bio-safety cabinet. Thin and thick smears were made by expressing 10 µl of blood on the slide, and use another clean slide held at 45 degrees to make thin films. The slides were left to air dry in the bio-safety cabinet, fixed with methanol for 5 minutes and stained with Giemsa stain 1 in 10 dilutions for 20 minutes. The smears were left to dry in an upright position in a bio-safety cabinet before they were examined under the compound microscope using 100× objective lens to confirm the presence of the *Plasmodium falciparum* parasites.

Caps were then removed from the flask containing medium, and 4.5 ml of medium was aspirated into the serological pipette and expelled into the 25 cc culture flasks. The red cells were re-suspended and the bottles gently agitated in a circular motion. The flasks were flushed with 5 % CO_2, 90 % N_2 and 5 % O_2 gas mixture and then placed at 37 °C in an incubator under moisture condition.

3.6.4 *Plasmodium falciparum* parasitemia in thin blood smear

A volume of 10 µl of blood was obtained by micropipette and transferred onto clean, sterile glass slides. Thin smears were made, air dried and fixed with 70 % methanol underflow laminar cabinet. The slides were stained using 1 in 10 dilutions of Giemsa stain for 20 minutes. Washed under running tap water and then allowed to air dry inside safety bio cabinet. Once the slides were dried, they were mounted on the microscope stage, and an oil emulsion applied with 100× objective lens. Three fields were randomly selected and observed and on each field count, the number of red blood cells and the number of parasites were counted up to 2000. Parasitaemia was calculated as the number of parasites divided by the total number of red blood cells.

3.6.5 Preparation of the splits (drugs, medium and culture)

Determination of necessary volume of culture was done, and 50 % fresh red blood cell (RBC) and medium needed for 5ml, 6 % haematocrit was done. The required volumes of

50 % red blood cells and medium in 25 cc culture flasks were mixed. The containers were placed in 37 °C dry incubator for 5 - 10 minutes. Warmed flasks plus old cultures were diluted in the laminar flow cabinet before new bottles were labeled. The desired volume of old culture was added into the corresponding flasks containing fresh red blood cells and medium mixed and placed horizontally on a working surface. Flasks were flushed with 5 % CO_2, 90 % N_2 and 5 % O_2. The new cultures were again mixed in a circular motion and placed horizontally in the 37 °C incubator.

3.6.6 Preparation of standard drugs (chloroquine and mefloquine)

This was done under a bio-safety cabinet. Stock solutions of 5 mg/ml were prepared in 5 ml of 100 % dimethyl sulfoxide (DMSO) for chloroquine (CQ) and mefloquine (MQ) respectively. CQ = 5 mg/ml × 1000000 = 5000000 ng/ml. Starting concentration on the plate was reduced to 2000 ng/ml. Therefore 5000000 ÷ 2000 = (1: 2500) × 9 = 9 µl in 2500 µl, hence 4 × (9 × 2500) = 36 µl in 10000 µl divided by 1000 hence chloroquine 36 µl in 10 ml medium. Approximately 300 µl of CQ was picked and transferred into the 1st well of the 1st column of 96 well microtiter plates.

The same procedure was repeated with MQ with the starting concentration of the plate = 500 ng/ml. 5000000 ÷ 500 =10000 hence 9 × (1µl in 10 ml) = 9 µl in 10 ml. About 300 µl of MQ was picked and transferred into the 2nd well of the1st column of the 384 microtiter plate. Further dilutions were done in complete medium with serum (150 µl) to reach the desired starting concentration of 2000 ng/ml and 500 ng/ml for CQ and MQ respectively. A serial 2-fold dilution followed to generate 10 concentrations for IC_{50} testing. Concentrations range from the highest to lowest were 2000 ng/ml to 1.977 and 500 ng/ml to 0.244 ng/ml for CQ and MQ respectively

3.6.7 Preparation of (methanolic and aqueous) extracts

Both extracted sample with methanol and aqueous were weighed using electronic analytical balance. The various weights obtained were dissolved in 100 % dimethyl sulfoxide (DMSO) and vortexed under laminar flow cabinet using Barnstead Lab-Line shaker / Thermolyne 2555 Bonlevard made in the USA. The amount of DMSO was weight dependent (Table 5). Starting concentration 10,000,000 ng/ml was reduced to 50,000 ng/ml. 10,000,000 ng/ml was divided by 50,000 ng/ml to obtain a ratio of 1: 200. Both sides of the ratio were multiplied by 10 to obtain a ratio of 10 µl of cpd: 2000 µl of complete medium with serum and cpd, 10 µl in 2 ml, see Table 6.

Table 6: Extracts identity, weight of extracts, and amount of solvent used to dissolve the extracts

Plant species	Extracts identity		Weight of extracts	Amount solvent
1. *C. edulis*	A	methanolic	5.5 mg	550 µl
2. *C. edulis*	A	aqueous	5.2 mg	520 µl
3. *A. indica*	B	methanolic	5.1 mg	510 µl
4. *A. indica*	B	aqueous	6.6 mg	660 µl
5. *C. siamea*	C	methanolic	5.5 mg	550 µl
6. *C. siamea*	C	aqueous	5.3 mg	530 µl
7. *H. abyssinica*	D	methanolic	6.7 mg	670 µl
8. *H. abyssinica*	D	aqueous	5.1 mg	510 µl

Methanol was used as an organic solvent and aqueous as inorganic solvent for the four medicinal plants. Key: A = *Carissa edulis*; B = *Azadirachta indica*; C = *Cassia siamea* and D = *Harrisonia abyssinica*. .

3.6.8 Preparation of primary culture (mother) plates

Approximately 300 µl of each reference and test drugs was added manually to the first well of column 1, 2, upto 8 of the 384 microtiter plates. Complete RPMI 1640 medium, 150 µl was added to well 2 of each coloum through 12. Biomek 2000 was used to perform two-fold serial dilutions by carrying 150 µl from first well of each column through 12.

3.6.9 Preparation of secondary culture (daughter) plates

Daughter plates were placed in the same orientation with the mother plate. A volume of 12.5 µl of each dilution per well was transferred from mother plate to daughter plate. Changing tips after every dispensation. The mother and daughter plates were kept at -80 °C for one day. The plates were thawed at 37 °C in a culture incubator for 1 - 2 hours before used for drug screening.

3.6.10 Setting up *in vitro* drug test

Crude extracts of *Carissa edulis* Vahl., *Azadirachta indica* A. Juss, *Cassia siame*a Lam., and *Harrisonia abyssinica* Oliv., were tested against chloroquine-sensitive and mefloquine- resistant designated as (CQ-S; MQ-R) hence (3D7) and chloroquine resistant and mefloquine-sensitive designated as (QC-R; MQ-S) hence (W2) strain of *P. falciparum* using anon - radioactive assay technique, (**S**milkstein *et al*., 2004) with modifications. This method used fluorochrome called ''SYBR Green I'', a non-radioactive DNA dye that accurately depicts *in vitro* parasite replication. The parasites were cultured to establish replication robustness and to attain a 3 - 6 % parasitemia. Concurrently, a serial dilution of the drugs chloroquine (2000 ng/ml - 1.977 ng/ml), mefloquine (500 ng/ml - 0.244 ng/ml) and test sample (50,000 ng/ml - 48.83 ng/ml) were prepared on a 384 well plate. The culture - adapted *P. falciparum* were reconstituted to 1 % parasitemia and added on to the plate containing dose range of the reference drugs and test samples and incubated in gas mixture (5 % CO_2, 5 % O_2, and 90 % N_2) at 37 ^oC. The assay was terminated 72 h later by freezing at - 80 ^oC for 24 h. After thawing, lysis buffer containing SYBR Green I (final concentration) was added directly to the plates and gently mixed by using the Beckman Coulter Biomek 2000 automated laboratory workstation (Beckman Coulter, Inc., Fullerton, CA). The plates were incubated for 5 - 15 minutes at room temperature in the dark, (Johnson *et al*., 2011).

3.7 Objective 3: Determination of cytotoxicity of *Carissa edulis*, *Azadirachta indica*, *Cassia siamea,* and *Harrisonia abyssinica* methanolic extracts on brine shrimp larvae (*Artemia salina nauplii*).

3.7.1 Brine shrimp bioassay

The brine shrimp larvae (*Artemia salina nauplii*) are use in the laboratory bioassay for toxicity through estimation of medium lethal concentration. Brine shrimp toxicity (BST) tests have been used as bench top pure bio-assay for the discovery and purification in research of natural products. Brine shrimp toxicity test is a low-cost test indicative of cytotoxicity activity. It is used to measure the concentration required to kill half of a group of shrimp $LC_{50,}$ (Ayo *et al*., 2007).

Artificial sea water was prepared by dissolving 18.0 g of brine shrimps eggs in a beaker containing half a litre of distilled water. The beaker containing 500 ml of distilled water and brine shrimp eggs were left at 25 °C temperature for 48 hours to allow the eggs to hatch, (Akeng'a, 2005).

3.7.2 Preparation of methanolic extracts of *Carissa edulis*, *Azadirachta indica, Cassia siamea* and *Harrisonia abyssinica* stock solutions.

Using analytical balance, 3mg of each methanolic extract was weighed and transferred into four different universal bottles containing 3ml of 1% dimethyl sulfoxide (DMSO). The preparation was kept in the refrigerator at 4 °C to be used for Brine shrimp toxicity bioassay test (Appendix 5c, Laboratory procedure).

A serial dilution (1000 µg/ml-31.25 µg/ml) of the methanol extracts of *Carissa edulis* Vahl; *Azadirachta indica* A. Juss*, Cassia siamea* Lam and *Harrisonia abyssinica* Oliv was prepared using 1 % dimethyl sulfoxide (DMSO). Each concentration was tested in triplicate. Dimethyl sulfoxide 1 % solution was used as a negative control. By the help of a sterile Pasteur pipette, brine shrimp larvae (10 life *nauplii*) were picked and added to each microtiter plate wells containing a concentration of methanolic crude extract. The microtitre plates were covered with perforated alluminium foil and incubated at room temperature for 24 hours. The cultured microtitre plate was placed on the dissecting microscope stage after incubation period and the *nauplii* responded to treatment viewed out. The number of dead and live brine shrimp larvae were confirmed by the help of an inverted microscope and recorded. The viewing and the counting of the dead *nauplii were* repeated after 48 hours. The brine shrimp lethality was expressed in percentage by; $\frac{\text{Number of shrimps - life shrimps}}{\text{Number of shrimps.}} \times 100\ \%$

Brine shrimps bioassay plate seeded with 10 *Artemia salina nauplii* in triplicate in a serial dilution from lowest to highest dilution (1000 µg/ml - 31.25 µg/ml) and 1 % DMSO, see plate 4.

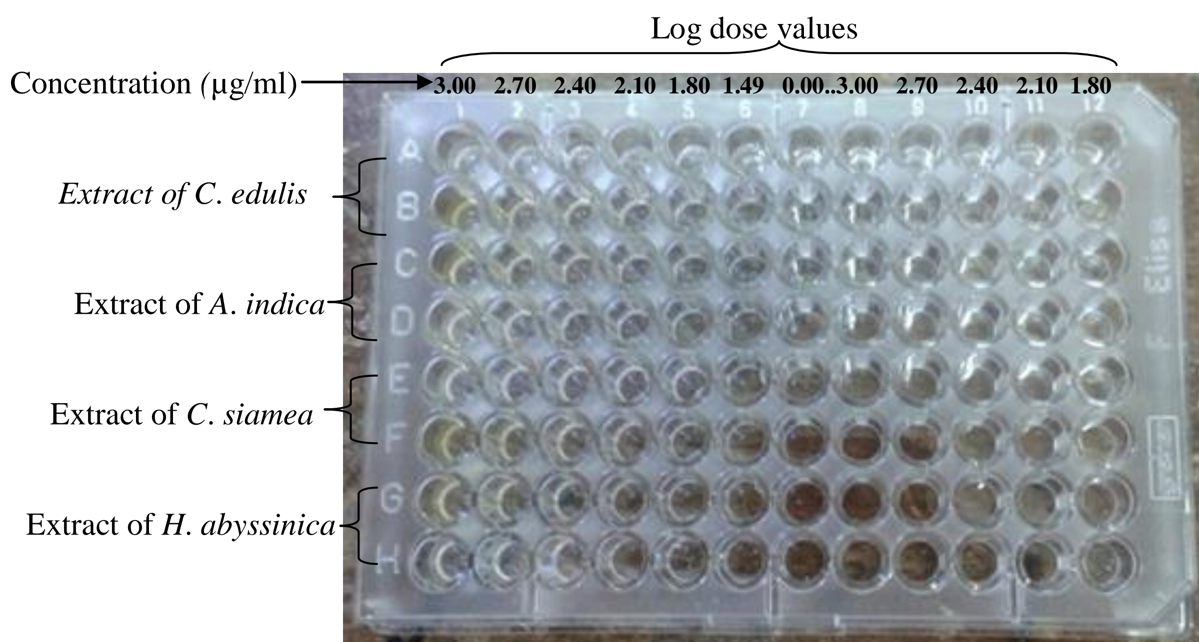

Plate 4: Brine shrimps bioassay plate seeded with 10 *Artemia salina nauplii* in triplicate in a serial dilution from lowest to highest dilution (1000 µg/ml - 31.25 µg/ml) and 1 % DMSO.

3.10 Data analysis

The phytochemicals of the plant extracts were analyzed using descriptive analysis; positive sign indicated the presence and negative indicated absence of the phytochemicals. Parasite growth inhibition was quantified by measuring the per-well relative fluorescence units (RFU) of SYBR Green 1 dye using the Tecan Genios Plus (Tecan US, Inc., Durham, NC) with excitation and emission wavelengths of 485 nm and 535 nm, respectively, and with the gain set at 60. Fluorescence is generated when a substance absorbs high energy, light with a short wavelength and emits low energy at a longer wave length. The IC_{50} for each drug calculated by anequation generating a sigmoidal concentration-response curve (variable slope), with log-transformed drug concentrations on the X-axis and relative fluorescence units (RFUs) on theY-axis (GraphPad Prism for Windows, version5.0; Graphpad Software, Inc., San Diego, CA. Raw data was copied from the exel sheetspecialpasted onto GraphPad file. Data was then transformed into sigmoidal graphs, (Juma *et al.,*2011).

Raw data obtained from brine shrimp bioassay was entered into the excel data sheet and analyzed using two ways ANOVA to determine the effect of toxicity and interactions of methanolic extracts of *Carissa eduli* root bark, *Azadirachta indica* leaves*, Cassia siamea* stem bark, and *Harrisonia abyssinica* root bark on *Artemia salina nauplii*. The LC_{50} of

cytotoxicity of each methanolic extract was entered into Excel spreadsheet and the output analyzed using EPA probit analysis version 1.5

Lethal concentration, Lethal dose and Inhibitory concentration (LC_{50}, LD_{50} and IC_{50}) are similar in the context of their measurements. IC_{50} is usually used for chemical assay or colorimetric or fluorimetric assays where one chemical concentration inhibits 50 % of these processes (e.g., RFUs measurements for plasmodial growth inhibition). Where as LC/LD_{50} are applicable to animals (e.g., rats and mice). LC_{50} is used to determine the concentration of a chemical in the air or water exposed within stipulated time to kill 50 % of the population. LD_{50} is to represent the drugs identical which kill 50 % population of animals in a single oral administration.

CHAPTER FOUR

RESULTS

4.1 Phytochemicals from the methanolic extracts

In this study, seven methanolic phytochemicals were identified namely (steroids saponins, glycosides, tannins, flavonoids, terpenoids and alkaloids).Tannins were found to be abundant in *Carissa edulis* root bark and *Azadirachta indica* leaves where as flavonoids were found to be abundant in *Azadirachta indica* leaves. Glycosides were the least present in the extracts of *Carissa edulis* Vahl., root bark, *Cassia siamea* Lam. stem bark, and *Harrisoniaabyssinica* root bark, see Table 7.

Table 7: Phytochemical groups of methanolic extracts of *C.edulis*, *A. indica*, *C. siamea* and *H. abyssinica*

Methanolic extracts	Chemical groups						
	Steroids	Saponins	Glycosides	Tannins	Flavonoids	Terpenoids	Alkaloids
C. edulis root bark	+	+	-	+++	+	++	-
A. indica leaves	++	+	+	+++	+ ++	+	++
C. siamea stem bark	+	+	-	+ +	-	+	+
H. abyssinica root bark	++	+	-	+	+	+	-

Key: Absence of colouration (-), presence of slight colouration (+), presence of deep colouration (++) and presence of very deep colouration (+++).

4.2 *In vitro* antiplasmodial activities

4.2.1 *In vitro* antiplasmodial activities of chloroquine, mefloquine, and methanolic and aqueous plant extracts on *Plasmodium falciparum* 3D7 strain

The successive rate of the control drugs were higher compared to those of the extracts, (e.g. for 3D7 *Plasmodium falciparum* strain, chloroquine, n = 29, mefloquine, n = 25, *Carissa edulis* methanolic extract, n = 11, *Carissa edulis* aqueous extract, n = 12, *Azadirachta indica* methanolic extracts, n = 11, *Azadirachta* indica aqueous extracts, n = 6, *Cassia siamea* methanolic extracts, n = 10, *Cassia siamea* aqueous extract, n = 9, *Harrisonia abyssinica* methanolic extract, n = 13 and *Harrisonia abyssinica* aqueous extract, n = 7 with chloroquine showing the highest and aqueous extract of *Azadirachta indica* leaves, and

Harrisonia abyssinica root bark in *Plasmodium falciparum* W2 strain the least (Appendices 2 and 3). However the cut off values for chloroquine and mefloquine as control drugs are15 ng/ml and 10 ng/ml respectively. The antiplasmodial activities of the controls (chloroquine and mefloquine) drugs and (methanolic and aqueous) extracts. Chloroquine drug showed antimalarial activities from concentration 0.977 ng/ml – 15.625 ng/ml, see Fig. 3.

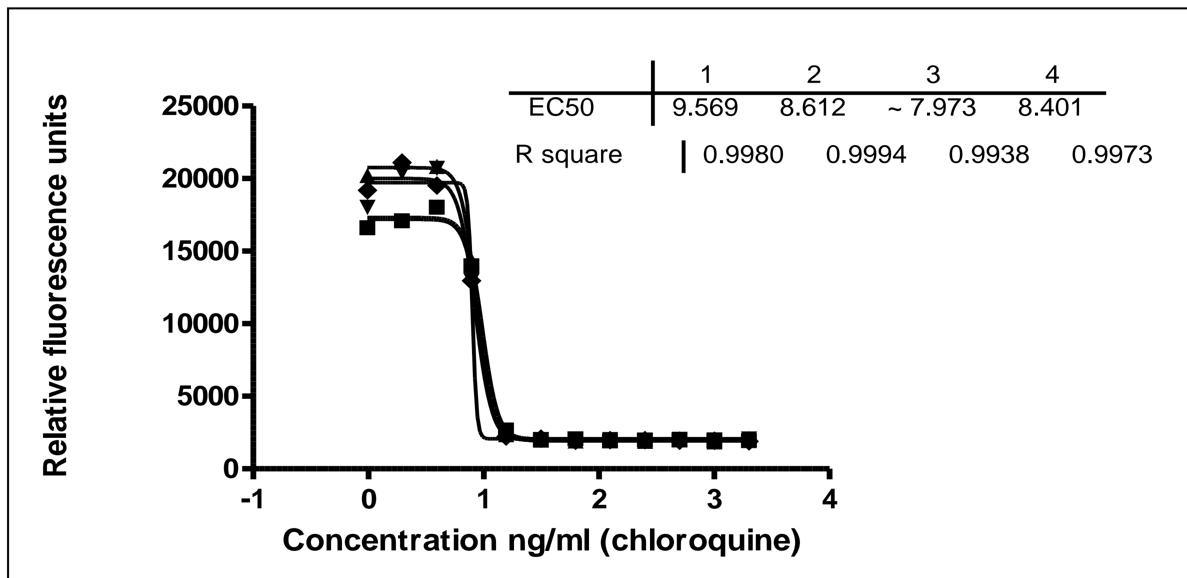

Figure 3: Antiplasmodial activities of chloroquine against 3D7

Key: 1, 2, 3 and 4 are replications; EC_{50} is Effective concentration of a drug which induces a response halfway between the baseline and maximum after a specified exposure of time.
R is reliability. Replication of chloroquine had EC_{50} value of 10, 9, and 8 ng/ml with reliability of 0.99.

Mefloquine drug showed antimalarial activities from concentration 0.244 ng/ml- 62.5 ng/ml, see Fig. 4.

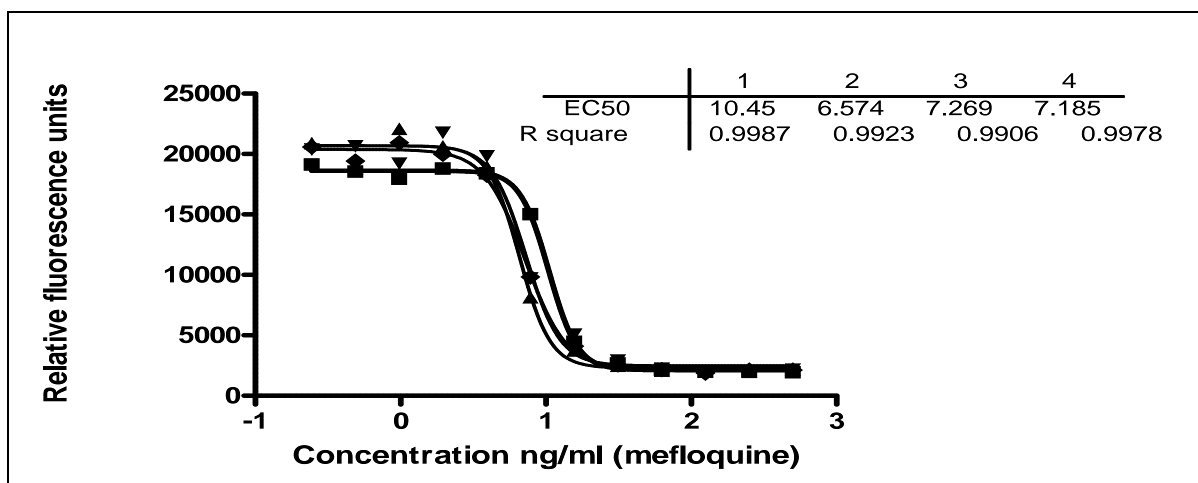

Figure 4: Antiplasmodial activities of mefloquine against 3D7

Key: 1, 2, 3, and 4 are replicates; EC_{50} is Effective concentration of a drug which induces a response halfway between the baseline and maximum after a specified exposure of time. R is reliability. Mefloquine had showed EC_{50} values of 10.1, 6.6, 7.1, and 7.2 with reliability values of 0.99.

Carissa edulis methanolic extracts showed antimalarial activities from concentrations 24.414 ng/ml – 25000 ng/ml, see Fig.5.

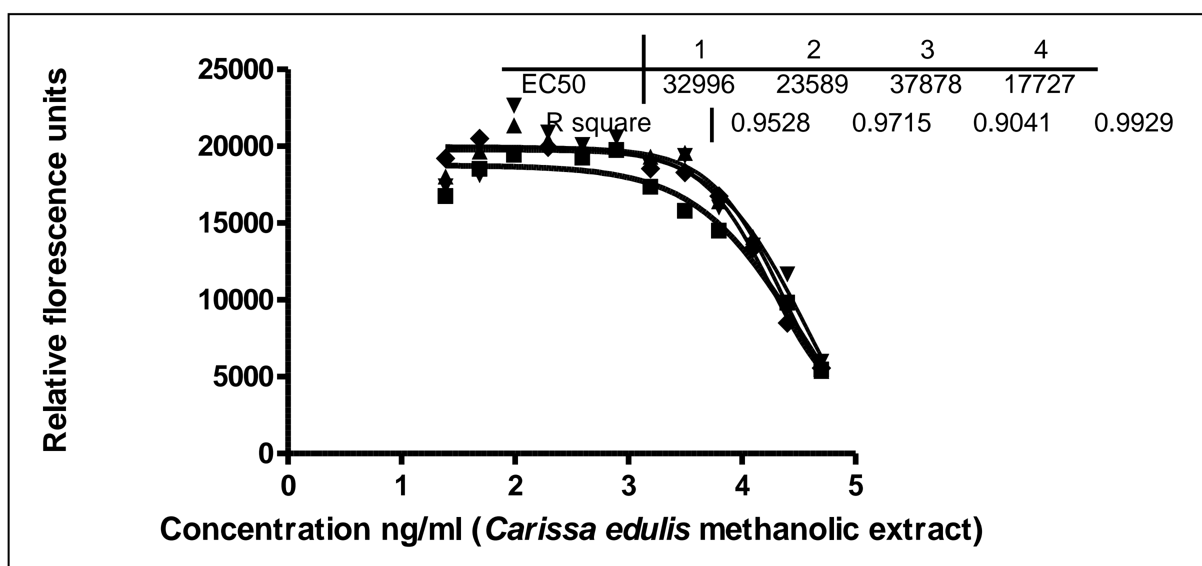

Figure 5: Antiplasmodial activities of *Carissa edulis* methanolic extracts

The extracts were replicated four times and since the graph was consistence, concentration would have been increased for the graph to exactly come out as that of the control. Although the replicate values were big (e.g., 32996, 23589, 37878, and 17727 ng/ml), because the extracts contains several compounds, they showed anti-plasmodial activities.

Carissa edulis aqueous extracts showed antimalarial activities from concentrations 24.414 ng/ml – 25000 ng/ml, see Fig.6.

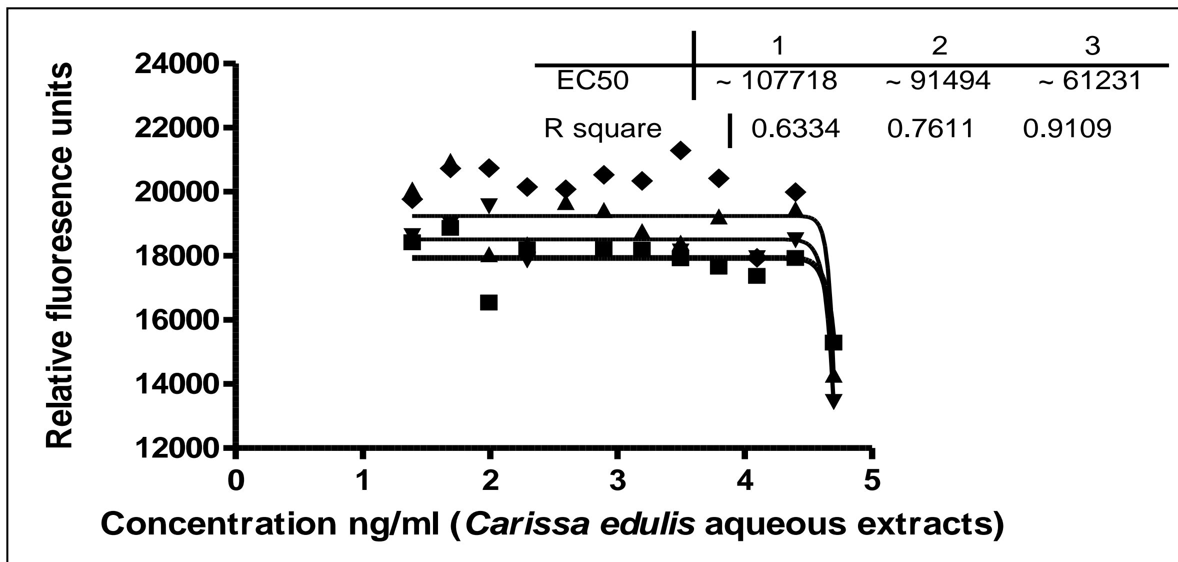

Figure 6: Antiplasmodial activities of *Carissa edulis* aqueous extracts

In *Carissa edulis* aqueous extracts only three out of four replicates performed anti-plasmodial activities with reliability values (e.g., 0.63, 0.76 and 0.91).

Methanolic extracts of *Azadirachta indica* showed antimalarial activities from concentrations 24.414 ng/ml – 25000 ng/ml, see Fig.7.

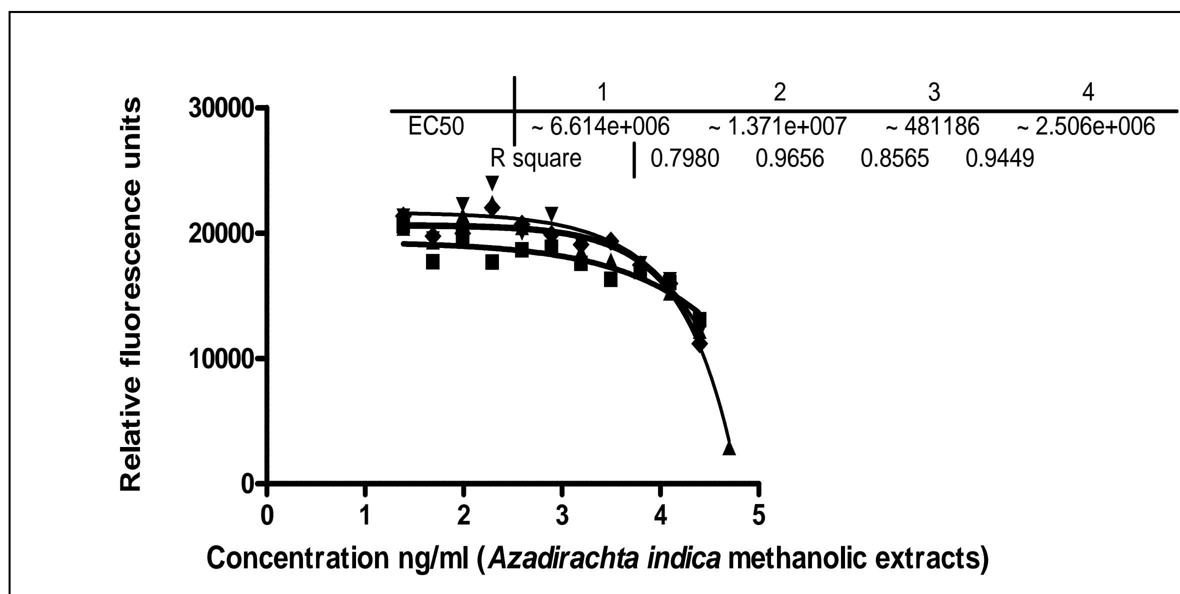

Figure 7: Antiplasmodial activities of *Azadirachta indica* methanolic extracts

In methanolic extracts of *Azadirachta indica* the four replicates showed antiplasmodial activities with EC_{50} values of 7, 1.4, 481186 and 2506 ng/ml, with reliability values of 0.80, 0.96, 0.86 and 0.94

Aqueous extracts of *Azadirachta indica* showed antimalarial activities from concentrations 24.414 ng/ml – 48.828 ng/ml, see Fig. 8.

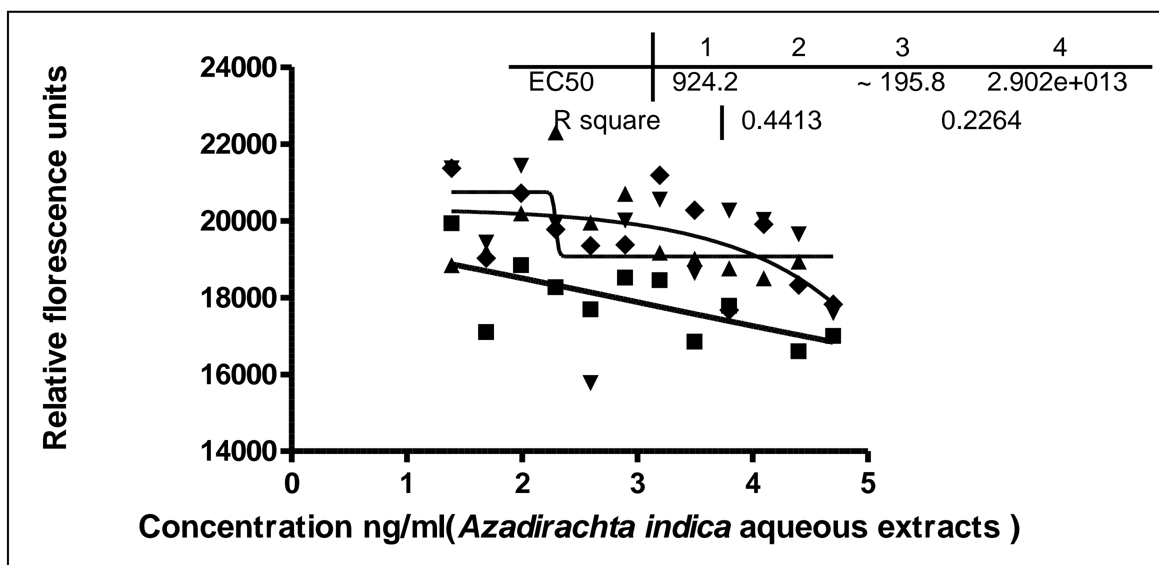

Figure 8: Antiplasmodial activities of *Azadirachta indica* aqueous extracts

In the aqueous of *Azadirachta indica*, three replicates demonstrated anti-plasmodial activities. The four replicates had EC_{50} values of 994, 196 and 2.9 ng/ml with reliability of 0.44 and 0.23.

Methanolic extracts of *Cassia siamea* showed antimalarial activities from concentrations 24.414 ng/ml – 48.828 ng/ml, see Fig. 9.

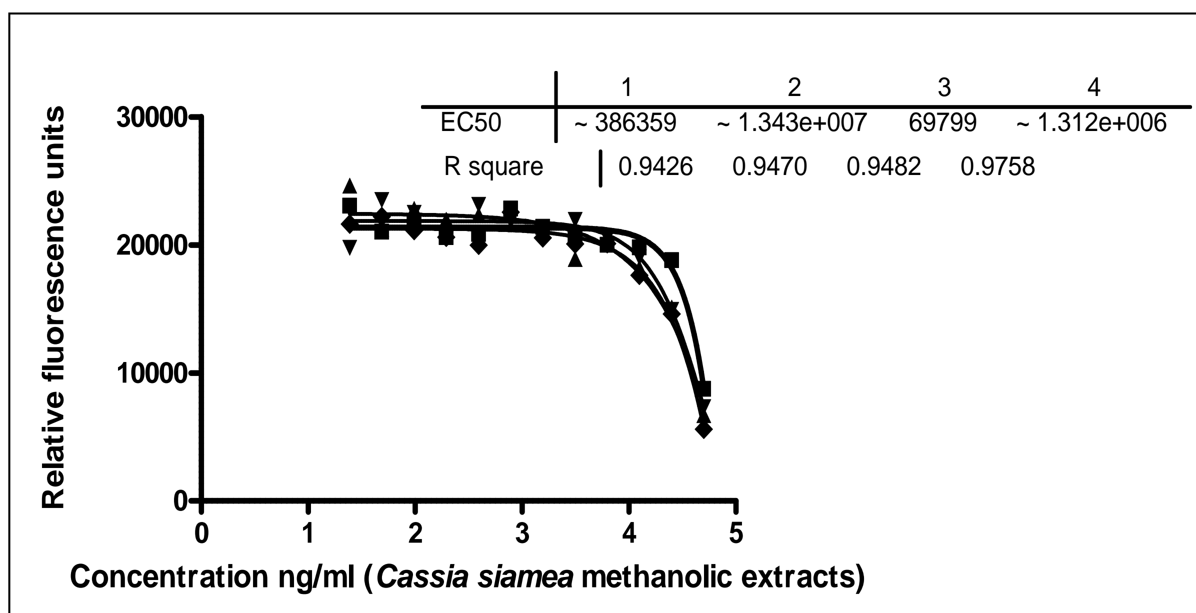

Figure 9: Antiplasmodial activities of *Cassia siamea* methanolic extracts

In methanolic extracts of *Cassia siamea* the four replicates demonstrated antiplasmodial activities with EC_{50} values of 386359, 1.34, 69799 and 1.3.with reliability values of 0.94, 0.95, and 0.98.

Aqueous extracts of *Cassia siamea* showed antiplasmodial activities from concentrations 24.414 ng/ml – 12500 ng/ml, see Fig.10.

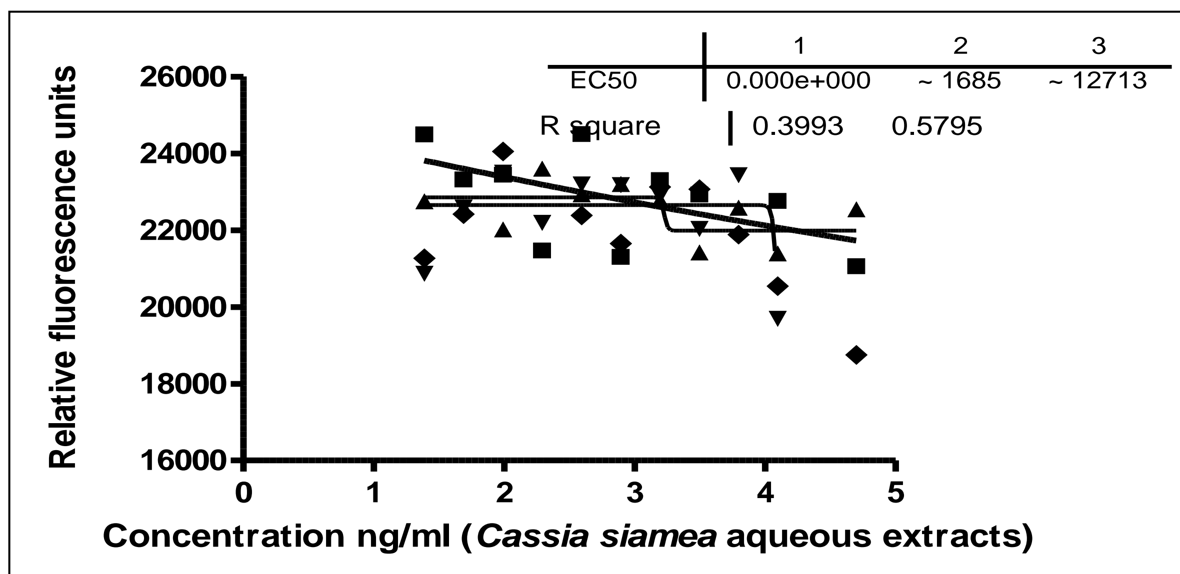

Figure 10: Antiplasmodial activities of *Cassia siamea* aqueous extracts

In the aqueous extracts of *Cassia siamea* two replicates showed antiplasmodial activities with EC50 values of 1689 and 12713 ng/ml and reliability values of 0.40 and 0.58.

Methanolic extracts of *Harrisonia abyssinica* showed antiplasmodial activities from concentrations 24.414 ng/ml – 12500 ng/ml, see Fig.11.

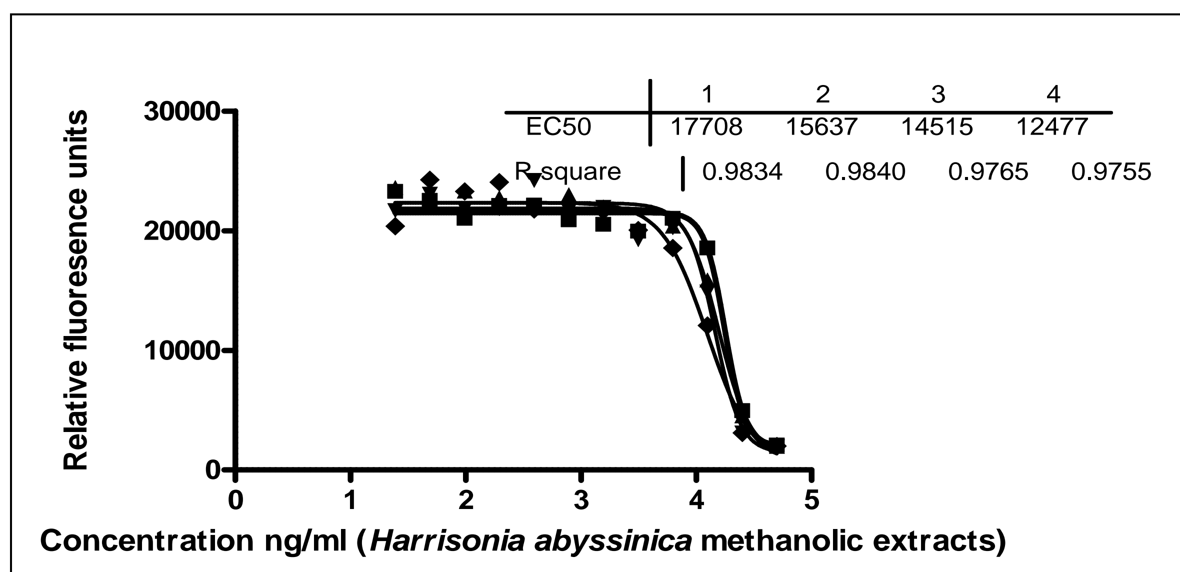

Figure 11: Antiplasmodial activities of *Harrisonia abyssinica* methanolic extracts

In methanolic extracts of *Harrisonia abyssinica* the four replicates showed antiplasmodial activities with EC_{50} values 17708, 15637, 14515, and 12477 ng/ml and reliability values of 0.98.

Aqueous extracts of *Harrisonia abyssinica* showed antiplasmodial activities from concentrations 24.414 ng/ml – 12500 ng/ml, see Fig. 12.

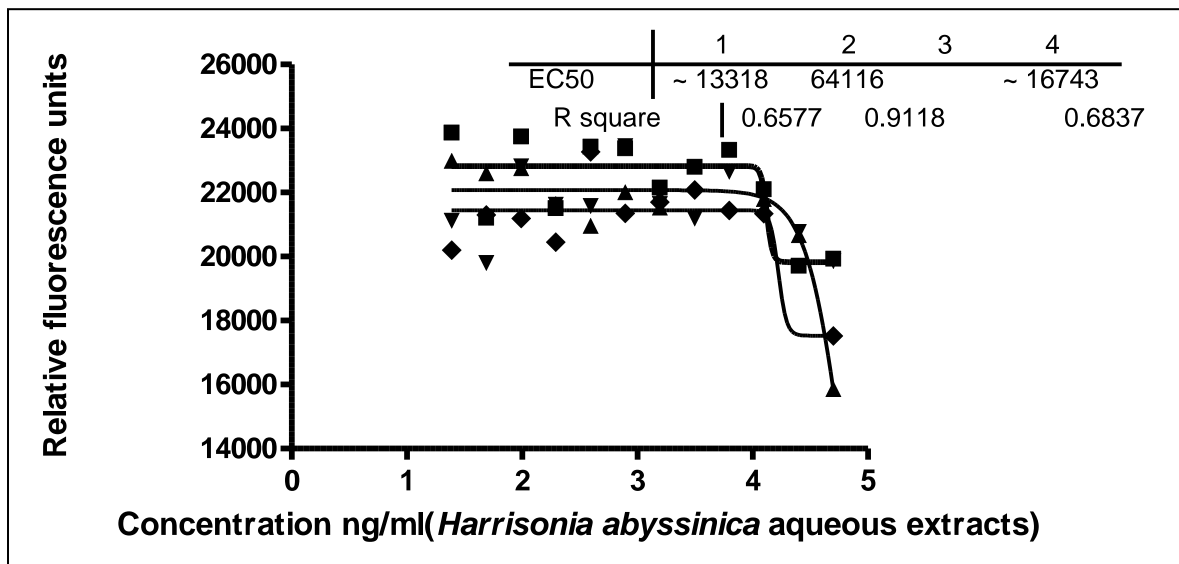

Figure 12: Antiplasmodial activities of *Harrisonia abyssinica* aqueous extracts

In the aqueous extracts of *Harrisonia abyssinica*, three replicates showed anti-plasmodial activities with EC_{50} values of 13318, 64116 and 16743 and reliability value of .0.66, 0.91 and 0.68.

4.2.2. Comparison of chloroquine, mefloquine and methanolic and aqueous extracts of the four plants on *Plasmodium falciparum* 3D7 strain

One-way analysis of variance and Kruskal-Wallis was used to compare mefloquine, chloroquine and methanolic and aqueous plant extracts on *P. falciparum* 3D7 strain, see Figures 13-15. $P = 0.001$.

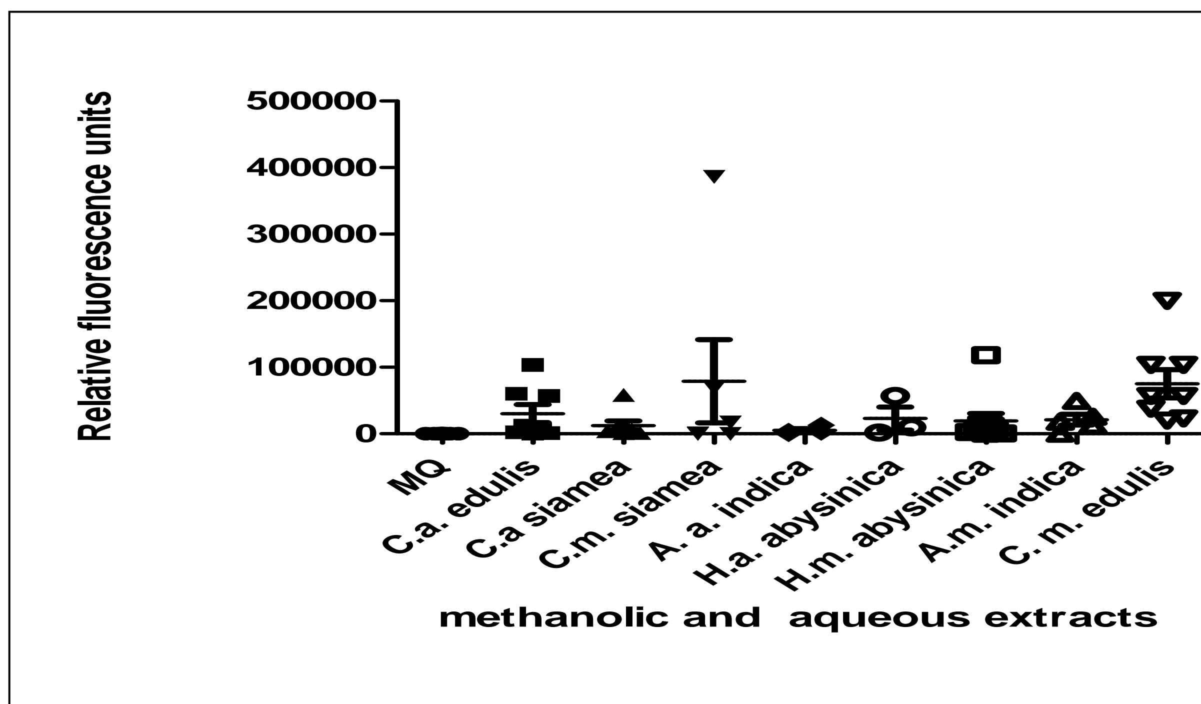

Figure 13: IC_{50} plots for mefloquine and methanolic and aqueous extracts against *P. falciparum* 3D7 strain RFUs

MQ is mefloquine and C.m, C.a, A.m, A.a, H.m, and H.a are methanolic and aqueous extracts of the four plants. All the methanolic and aqueous extracts of the four medicinal plants showed anti-plasmodial activities since they were on the same axis with the control drug, e.g., mefloquine. Methanolic extracts of *Cassia siamea*, *Azadirachta indica* and *Carissa edulis* as well as aqueous extracts of *Carissa edulis* and *Harrisonia abyssinica* showed 50 % inhibitory concentration on *Plasmodium falciparum* 3D7 strain.

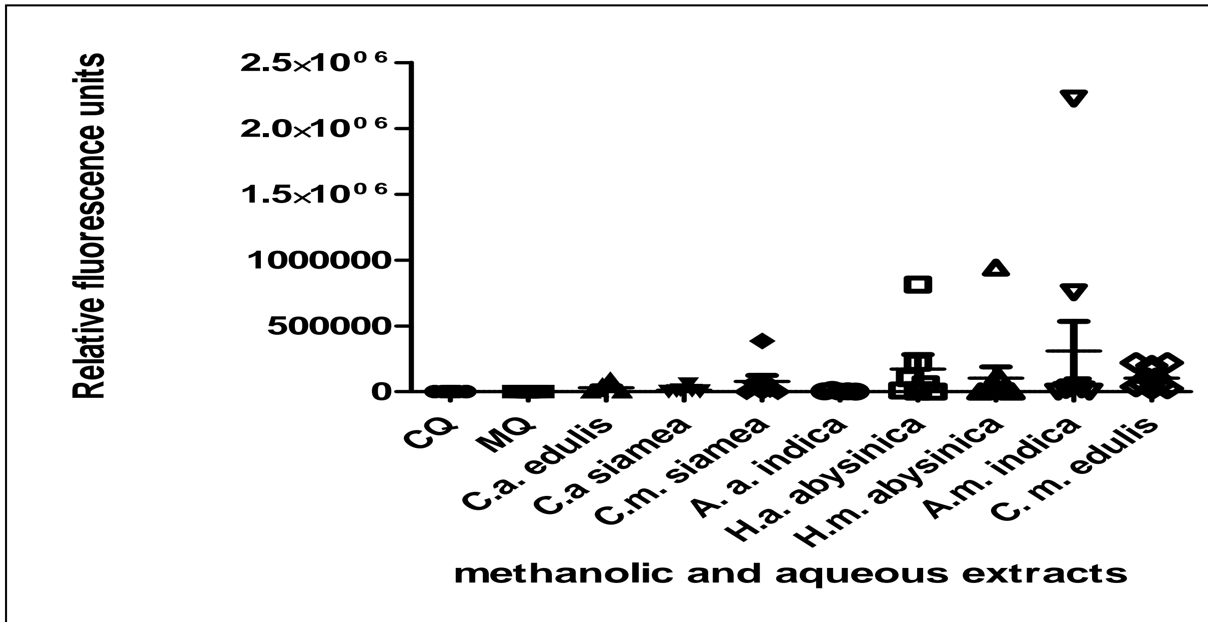

Figure 14: IC_{50} Plots for chloroquine, mefloquine and methanolic and aqueous extracts against 3D7 strain RFUs.

In chloroquine, mefloquine, methanolic and aqueous extracts of the four plants showed antiplasmodial activities against *Plasmodium falciparum* 3D7 strain. Methanolic extracts of *Harrisonia abyssinica* and *Azadirachta indica* as well as aqueous extracts of *Harrisonia abyssinica* showed 50 % inhibitory concentration.

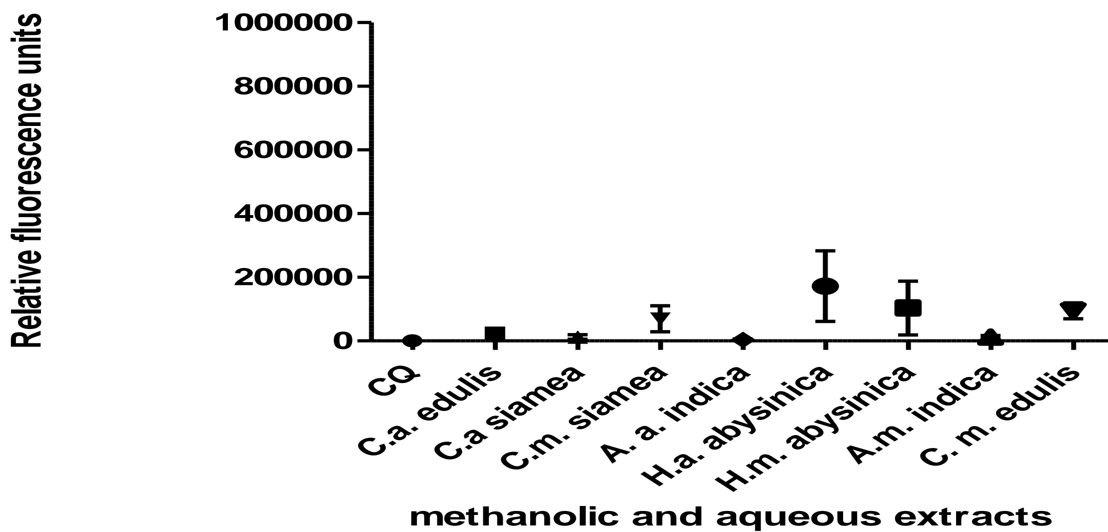

Figure 15: IC_{50} plots for chloroquine and methanolic and aqueous extracts against *P. falciparum* 3D7 strain RFUs.

In chloroquine, methanolic and aqueous extracts of the four plants against *Plasmodium falciparum* 3D7 strain, the extracts showed antiplasmodial activities. Methanolic extracts of *Cassia siamea* and *Harrisonia abyssinica*, as well as aqueous extracts of *Harrisonia abyssinica* showed 50 % inhibitory concentration on *Plasmodium falciparum* 3D7 strain.

4.3 *In vitro* antiplasmodial activities of chloroquine, mefloquine and methanolic and aqueous extracts on *Plasmodium falciparum* W2 strain

Chloroquine drug showed antimalarial activities *Plasmodium falciparum* W2 strain from concentrations 0.977 ng/ml –7.813 ng/ml, see Fig.16.

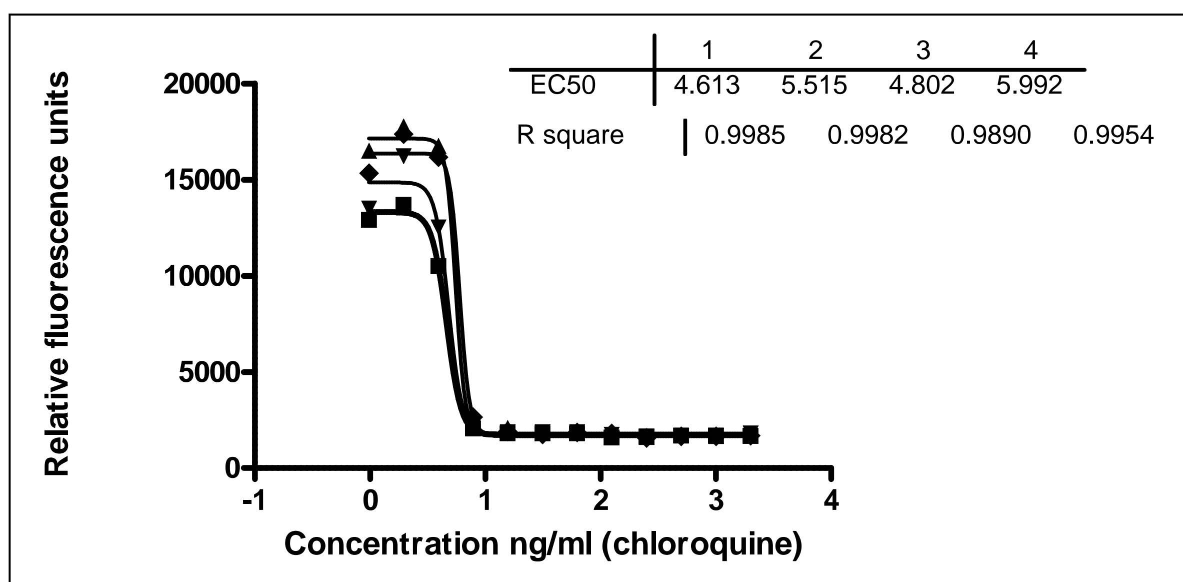

Figure 16: Antiplasmodial activities of chloroquine on W2

The four replicates of chloroquine showed antiplasmodial activities with EC_{50} values of 5, and 6 ng/ml with reliability value of 0.99 on W2 *Plasmodium falciparum* strain.

Mefloquine drug showed antimalarial activities on *Plasmodium falciparum* W2 strain from concentrations 0.244 ng/ml – 15.625 ng/ml, see Fig.17.

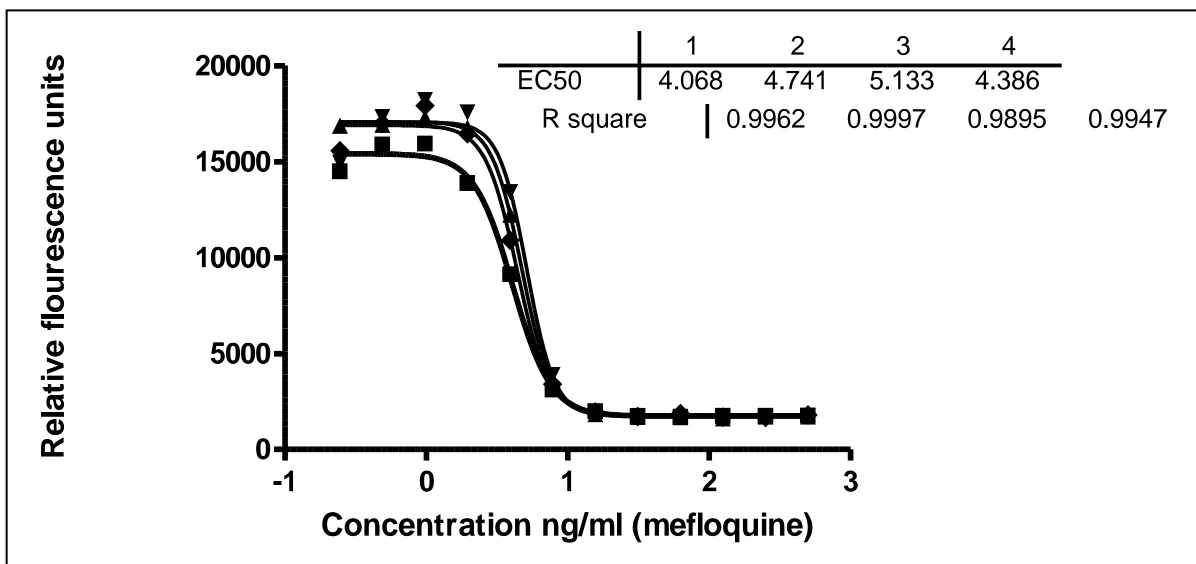

Figure 17: Antiplasmodial activities of mefloquine on *P. falciparum* W2 strain

The four replicates of mefloquine had antiplasmodial activities with EC_{50} values of 4.1, 4.8, 5.1, and 4.4 ng/ml with reliability values of 0.99.

Methanolic extracts of *Carissa edulis* showed antimalarial activities on *P. falciparum* W2 strain from concentrations 24.414 ng/ml - 25000 ng/ml, see Fig.18.

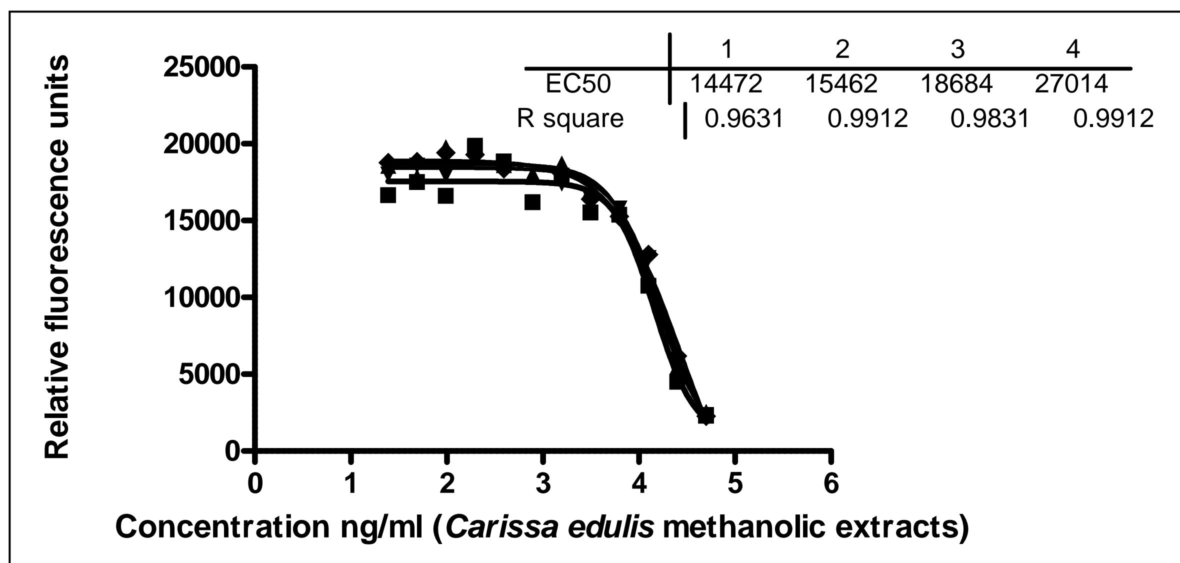

Figure 18: Antiplasmodial activities of *Carissa edulis* methanolic extracts

Carissa edulis methanolic extracts showed antiplasmodial activities with EC_{50} values of 14472, 15462, 18684 and 27014 ng/ml with reliability values of 0.96, 0.99, and 0.98.

Aqueous extracts of *Carissa edulis* showed antimalarial activities on *P. falciparum* W2 strain from concentrations 24.414 ng/ml - 25000 ng/ml, see Fig. 19.

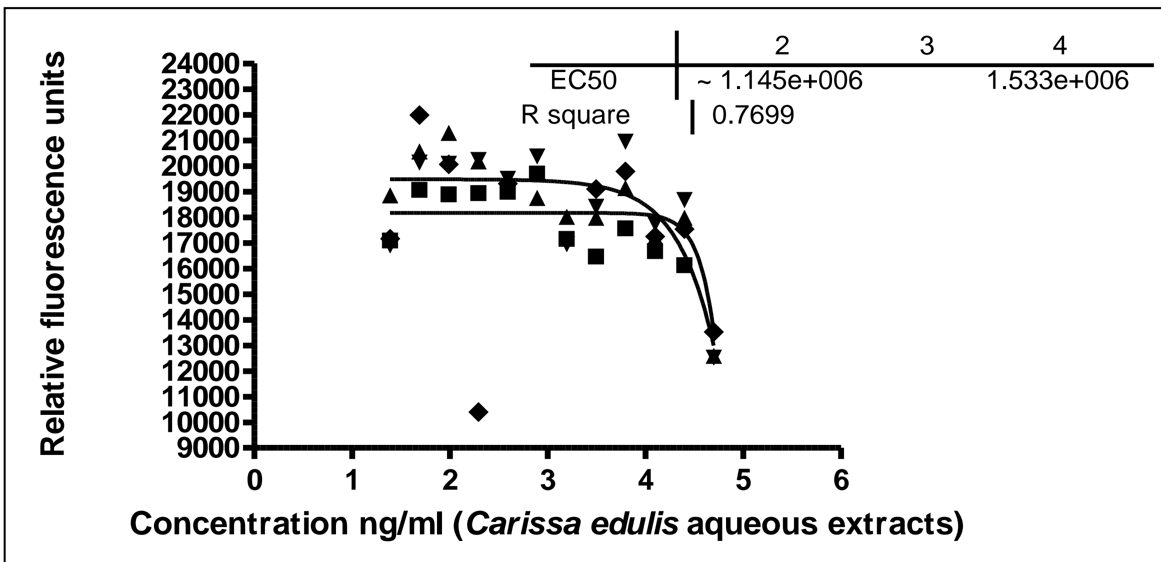

Figure 19: Antiplasmodial activities of *Carissa edulis* aqueous extracts

Aqueous extracts of *Carissa edulis* had antiplasmodial activities with EC_{50} values of 1.15 and 1.153 ng/ml and reliability of 0.77 .

Methanolic extracts of *Azadirachta indica* showed antimalarial activities on *P. falciparum* W2 strain from concentrations 24.414 ng/ml - 25000 ng/ml, see Fig. 20.

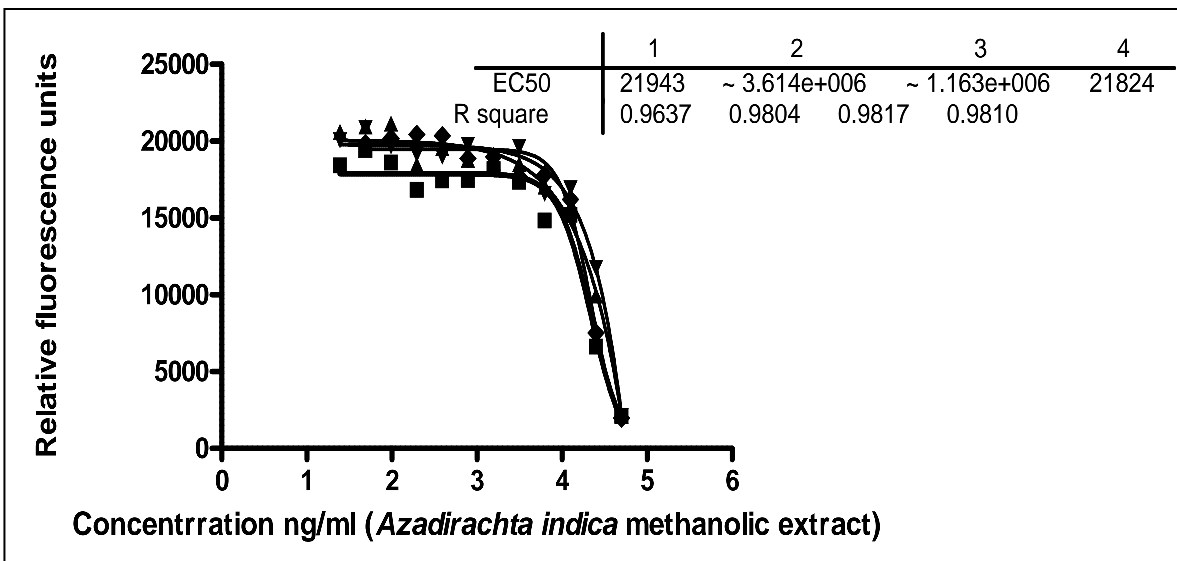

Figure 20: Antiplasmodial activities of *Azadirachta indica* methanolic extracts

Methanolic extract of *Azadirachta indica* showed antiplasmodial activities with EC_{50} values of 21342, 3.61, 1.16 and 21824 and reliability values of 0.96 and 0.98.

Aqueous extracts of *Azadirachta indica* showed antimalarial activities on *P. falciparum* W2 strain from concentrations 24.414 ng/ml -12500 ng/ml, see Fig. 21.

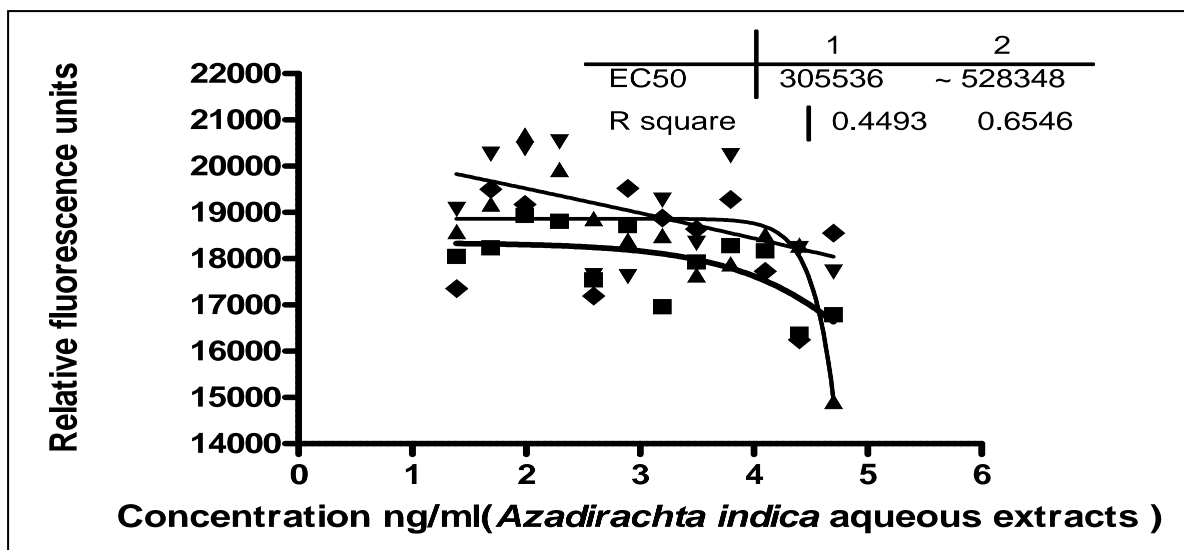

Figure 21: Antiplasmodial activities of *Azadirachta indica* aqueous extracts

Aqueous extracts of *Azadirachta indica* demonstrated antiplasmodial activities at EC_{50} values of 305536 and 528348 ng/ml with reliability value of 0.45 and 0.65.

Methanolic extracts of *Cassia siamea* showed antimalarial activities on *P. falciparum* W2 strain at concentrations 24.414 ng/ml - 25000 ng/ml, see Fig. 22.

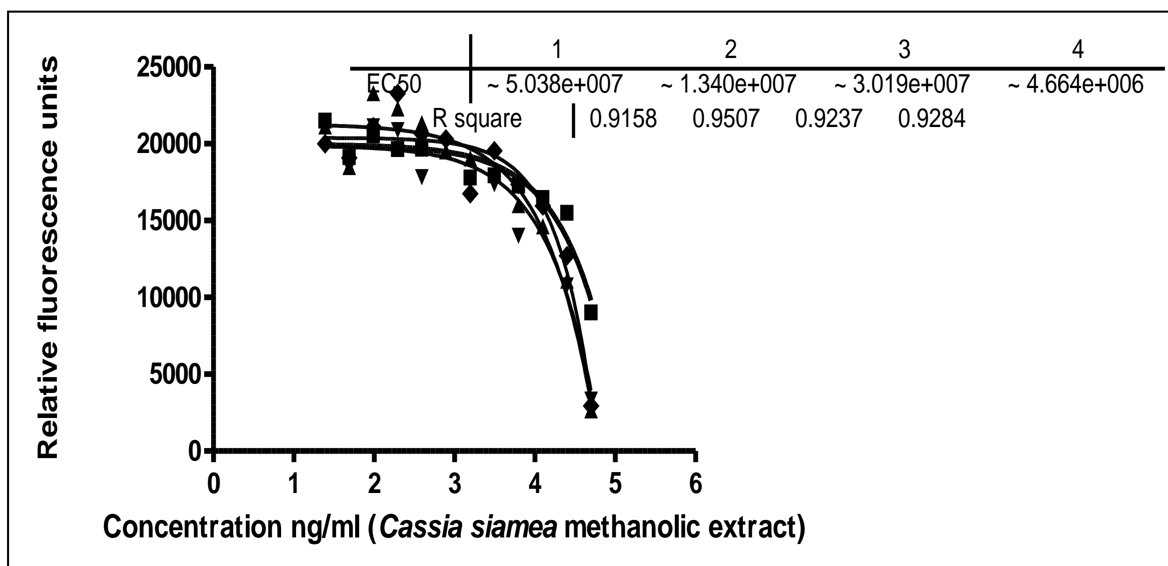

Figure 22: Antiplasmodial activities of *Cassia siamea* methanolic extracts

Methanolic extract of *Cassia siamea* showed anti-plasmodial activities at 5.04, 1.34, 3.02 and 4.67 ng/ml with reliability values of 0.92, 0.95, 0.92 and 0.93.

Aqueous extracts of *Cassia siamea* showed antimalarial activities on *P. falciparum* W2 strain at concentrations 24.414 ng/ml - 25000 ng/ml, see Fig. 23.

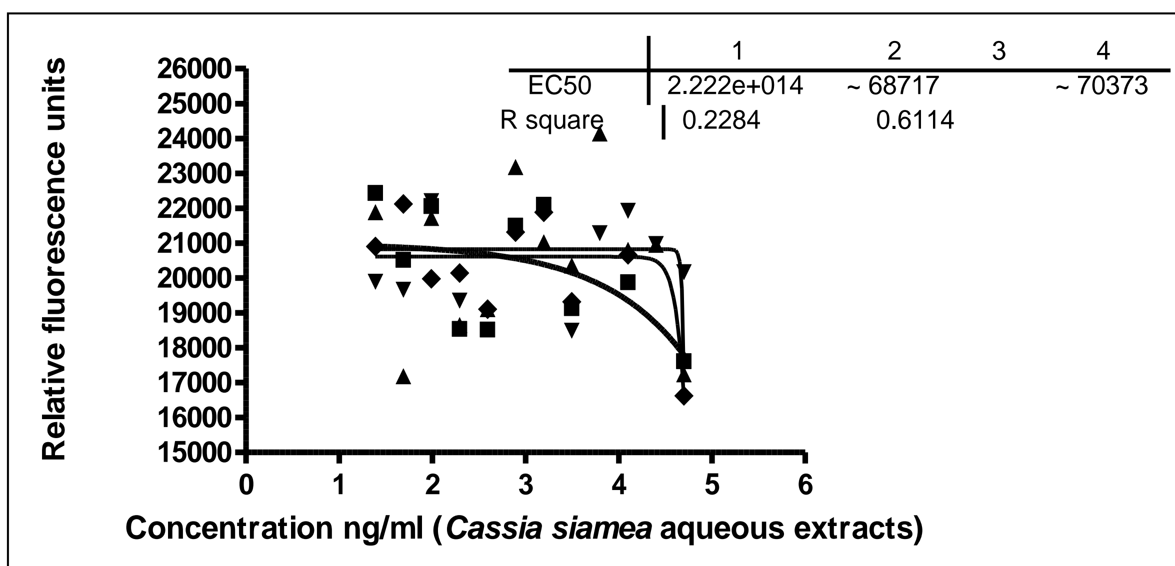

Figure 23: Antiplasmodial activities of *Cassia siamea* aqueous extracts

Aqueous extracts of *Cassia siamea* showed antiplasmodial activities with EC_{50} values of 2222, 68717 and 70373 ng/ml and reliability values of 0.23 and 0.61.

Methanolic extracts of *Harrisonia abyssinica* showed antimalarial activities on *P. falciparum* W2 strain at concentrations 24.414 ng/ml - 25000 ng/ml, see Fig. 24.

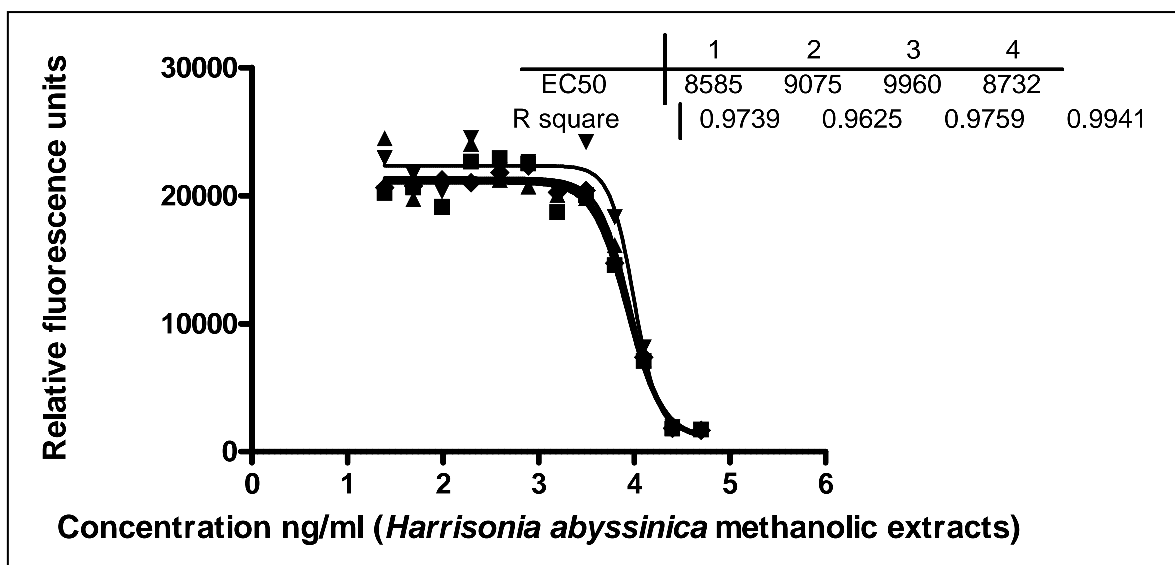

Figure 24: Antiplasmodial activities *Harrisonia abyssinica* methanolic extracts

Methanolic extracts of *Harrisonia abyssinica* showed antiplasmodial activities with EC_{50} values of 8585, 9075, 9960 and 8732 ng/ml and reliability values of .097, 0.96 and 0.98.

Aqueous extracts of *Harrisonia abyssinica* showed antimalarial activities on *P. falciparum* W2 strain at concentrations 24.414 ng/ml - 25000 ng/ml, see Fig. 25.

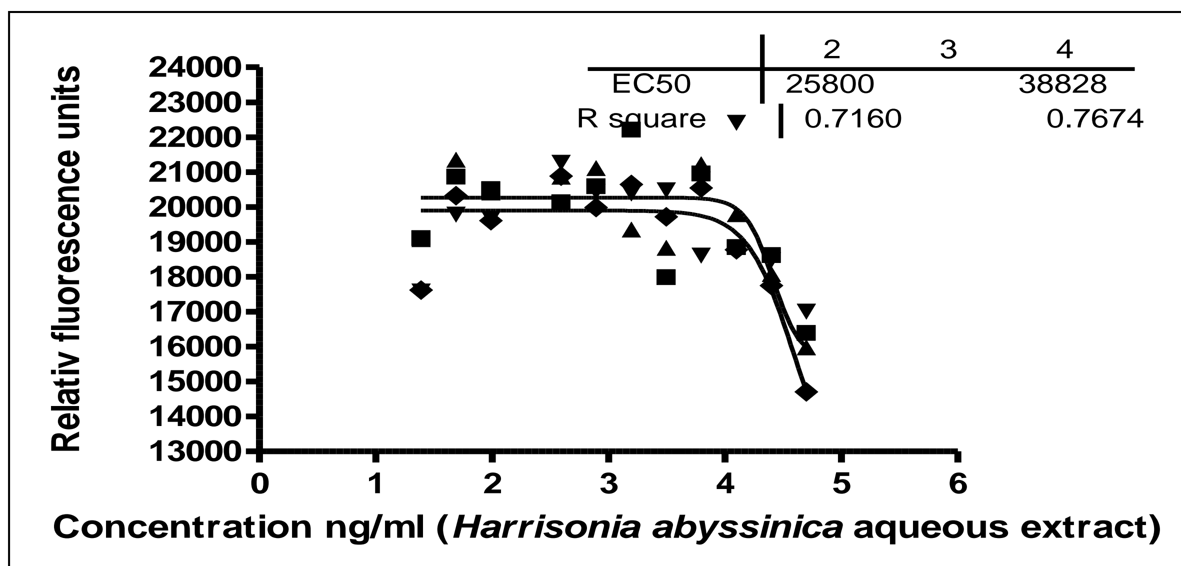

Figure 25: Antiplasmodial activities *Harrisonia abyssinica* aqueous extracts

Aqueous extracts of *Harrisonia abyssinica* showed anti-plasmodial activities with EC_{50} 25800 and 38828 ng/ml and reliability values of 0.71 and 0.76.

4.3.1 Comparison of chloroquine, mefloquine and extracts of the four (methanolic and aqueous) plants extracts on *Plasmodium falciparum* W2 strain

One-way analysis of variance (ANOVA) and Kruskal-Wallis was used to compare the control drugs (e.g., mefloquine and chloroquine) and methanolic and aqueous plant extracts against *Plasmodium falciparum* W2 strain, see Figs. 27- 28). ***, $P = 0.001$.

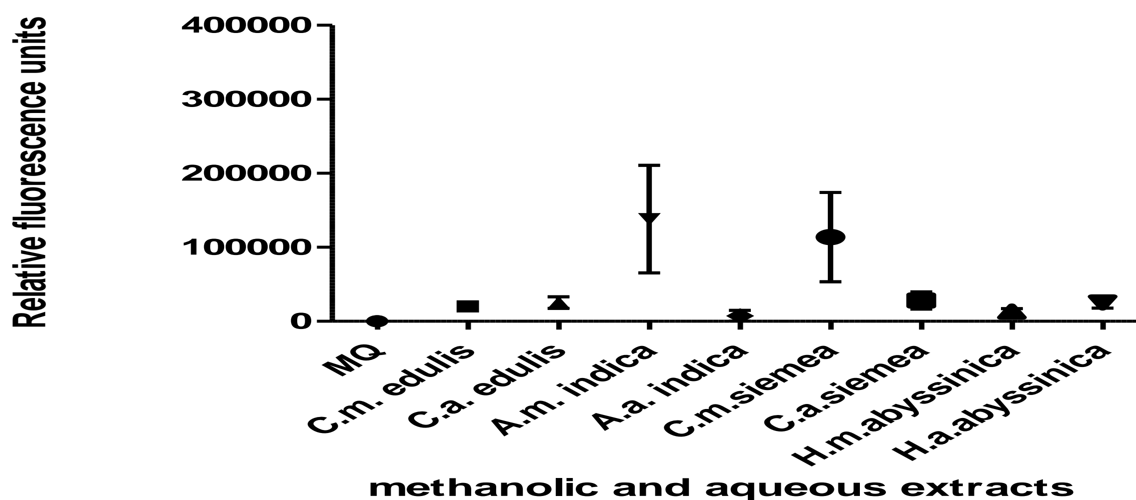

Figure 26: IC_{50} plots for mefloquine and methanolic and aqueous extracts against *P. falciparum* W2 strain RFUs

MQ is mefloquine and C.m, C.a, A.m, A.a, H.m, and H.a, are methanolic and aqueous extracts of the plants. Methanolic extracts of *Azadirachta indica* and *Cassia siamea* had shown 50 % inhibitory concentration similar to mefloquine on of *Plasmodium falciparum* W2 strain

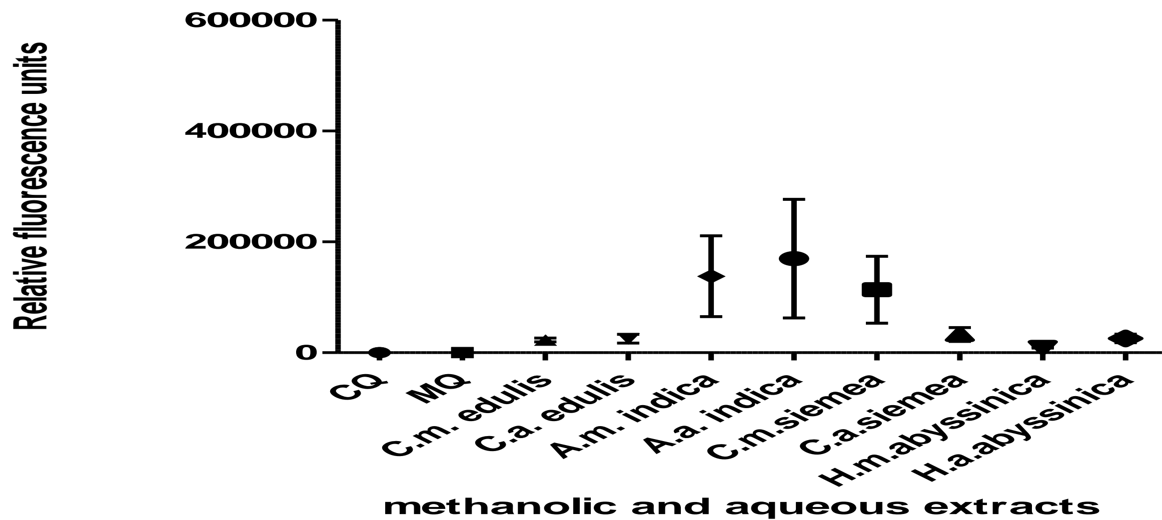

Figure 27: IC_{50} plots for chloroquine, mefloquine, and methanolic and aqueous extracts against *Plasmodium falciparum* W2 RFUs.

CQ is chloroquine, MQ is mefloquine and C.m, C.a, A.m, A.a, H.m, and H.a are methanolic and aqueous extracts of the plants. Methanolic extracts of *Azadirachta indica* and *Cassia siamea* as well as aqueous extracts of *Azadirachta indica* had showed 50 % Inhibitory concentration (IC_{50}) similar to that of chloroquine and mefloquine on *Plasmodium falciparum* W2 strain.

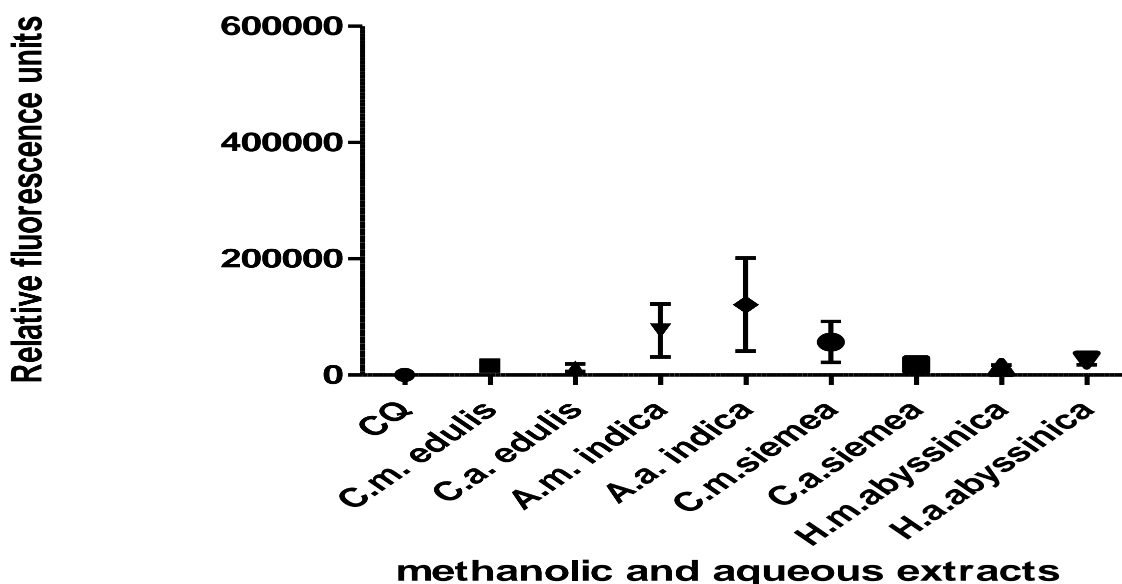

Figure 28: IC_{50} plots for chloroquine and extracts against *Plasmodium falciparum* W2 strain.

CQ is chloroquine, and C.m, C.a, A.m, A.a, H.m, and H.a are methanolic and aqueous extracts of the four medicinal plants**.** Methanolic extracts of *Azadirachta indica* and *Cassia siamea* as well as aqueous extracts of *Azadirachta indica* had showed 50 % Inhibitory concentration (IC_{50}) similar to that of chloroquine on *Plasmodium falciparum* W2 strain

4.4 Brine shrimp cytotoxicity bioassay

Brine shrimp toxicity test was considered a rapid inexpensive and sample bioassay for testing plant extracts lethal concentration which correlated with the cytotoxicity properties of the plants. It is used to measure the concentration required to kill half of a group of shrimps (LC_{50}).

The percentage death decreased with a decrease in drug concentration in all the four methanolic extracts (Appendix 4). The four methanolic extracts demonstrated different mortality rate at different concentrations. *Cassia siamea* methanolic stem bark extract demonstrated a higher concentration that was 500 µg/ml or (log concentration 2.7 mg/ml). In these results 77 % death of brine shrimps was demonstrated by methanolic extract of *Cassia siamea* stem bark. Methanolic leave extracts of *Azadirachta indica* demonstrated 67 % death of brine shrimps at log (concentration 2.4µg/ml). The same was demonstrated in methanolic extracts of *Carissa edulis* root bark which caused 64 % death of brine shrimps at log (conc.2.4 µg/ml). Methanolic extracts of *Harrisonia abyssinica* root bark gave 47 % death of brine shrimps at log (concentration 2.1µg/ml), see Figs. 29 and 30.

The concentration range of below 100 µg/ml was considered to have high cytotoxicity activity in all the four methanolic extracts. Values between 100 µg/ml - 500 µg/ml were found to be moderately toxicity. Concentration range of 500 µg/ml - 1000 µg/ml was considered to be weak cytotoxic activity, and any concentration value of more than 1000 µg/ml was found to be non-toxicity.

Cassia siamea stem bark methanolic extract was found to demonstrate no toxicity effect at progressively lower doses. Its concentration range was (250 µg/ml - 500 µg/ml with an LC_{50} value of 450.022. This showed that it has moderate cytotoxic activity. Both *Carissa edulis*root bark methanolic extracts and *Azadirachta indica* leaves methanolic extract were moderately toxic with concentration arrange of (250 µg/ml - 500 µg/ml and LC_{50} value of 255.606 and 233.061 respectively. Although *Carissa edulis* root bark methanolic extracts tend to be moderately toxic it showed less cytotoxicity activity compared to *Azadirachta indica* leaves methanolic extract at concentration, range of (250 µg/ml - 500 µg/ml). *Harrisonia abyssinica* root bark methanolic extracts had a concentration range of (125 µg/ml -250 µg/ml with LC_{50} value of 198.498), see Table 8.

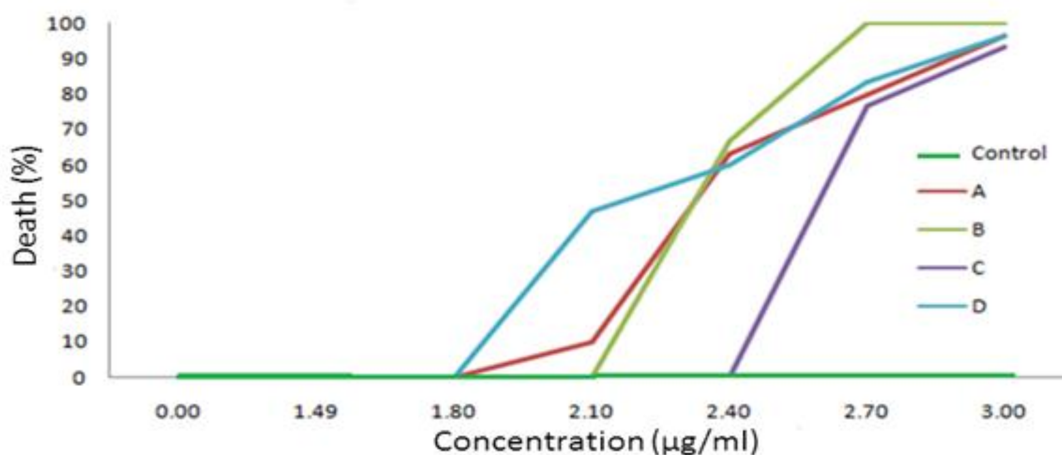

Figure 29: Percentage mortality values plotted against log dose. Key: A = *Carissa edulis*, B = *Azadirachta indica*, C = *Cassia siamea* and D = *Harrisonia abyssinica*

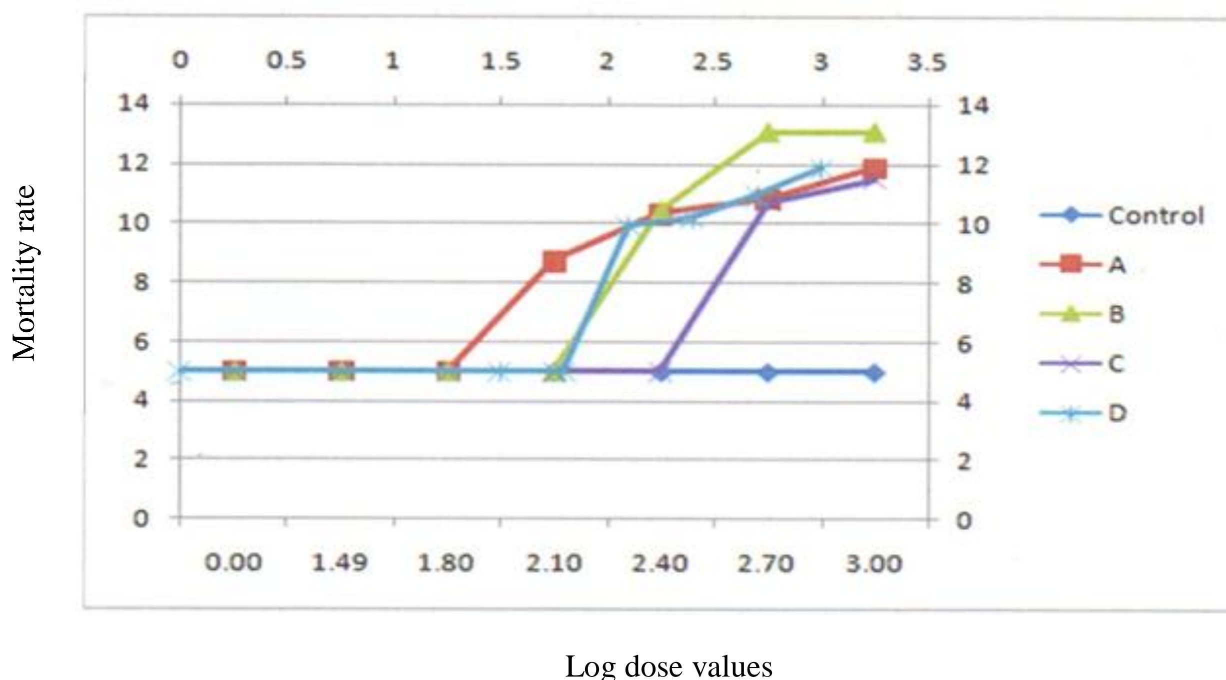

Figure 30: LC_{50} percentage mortality values converted to probit value against log doses. $LC_{50,}$ A = *Carissa edulis*, B = *Azadirachta indica*, C = *Cassia siamea* and D = *Harrisonia abyssinica*

Table 8: IC_{50} values for cytotoxicity of methanolic extracts of *C. edulis*, *A. indica*, *C. siamea* and *H. abyssinica* determined by EPA computer probit version 1.5 software.

Methanolic crude extracts	LC_{50} (µg/ml)	95% CI lower &	upper limit
Carissa edulis	255.606	212.354	303.185
Azadirachta indica	233.061	203.404	266.828
Cassia siamea	450.022	294.099	692.249
Harrisonia abyssinica	198.498	161.312	244.669

LC_{50} means the concentration that kills 50 % of the population in µg/ml; 95 % confidence interval gives the fiducial limit; CI represents confidence interval, lower and upper limit.

In the analysis derived from ANOVA (Appendix 1) at 5 % level of significance concentration 1000 µg/ml, demonstrated that, the effect was significant at ($P < 0.05$) while the interaction was significant at ($P = 0$). Concentration 500 µg/ml showed that the effect and interaction were significant at ($P < 0.05$). For concentrations 250 µg/ml, the effect was

significant at (P = 0) while the interaction was significant at (P < 0.05). Concentration 125 µg/ml, both the effect and the interaction were significant at (P < 0.05). There was no variation in concentrations 62.5 µg/ml and 31.25 µg/ml, see, Table 9.

Table 9: Significance of the concentrations of methanolic extracts of C.edulis, A. indica, C. siamea and H. abyssinica determined by Analysis of variance (ANOVA)

		Concentration (µg/ml)					
Source	df	1000	500	250	125	62.5	31.25
Extracts	3	0.00	0.00	0.00	0.00	0	0
Effect	1	522.67***	294.00***	1.50*	308.17***	600	600
Interaction	3	0.44*	6.44***	60.61***	29.50***	0	0
Error	16	0.50	0.67	2.17	1.33	0	0

Key: *, **, *** represents significance at P = 0.05, 0.01, 0.001 respectively.

CHAPTER FIVE

DISCUSSIONS

In this study, seven phytochemicals were identified namely; alkaloids, cardiac glycosides, flavonoids, saponins, steroids, tannins and terpenoids. Plants usually produce phytochemicals as an evolutionary adaptation to the harsh environment or in response to an attack by other organisms, (Awoyinka *et al*., 2007).

Tannins were identified in all the four methanolic extracts in this study. Tannins were abundant in *Carissa edulis* root bark and *Azadirachta indica* leaves. Tannins have a physiological role by acting as antioxidants free radical, scavenging activities, and chelation of transition metals. Inhibition of pro-oxidative enzymes and lipid peroxidation, (Ngoci *et al*., 2012). Inhibit tumor growth by inducing apoptosis, (Mohammed *et al*., 2012). Tannins exhibit anti-microbial activity by complexing proteins such as adhesins, substrates, cell wall and cell membrane proteins, thus inactivating microbial adhesion which is the first step in the establishment of infections, and also causing cell wall or membrane disruption, (Scalbert *et al*., 2005). Tannins are known to inactivate microbial enzymes and cell envelope transport proteins by processes that may involve reaction with sulfhydryl groups or through non-specific interaction with the proteins, (Biradar *et al*., 2007; Samy and Gopalakrishnakone, 2008). They are also anti-inflammatory, molluscicidal and hence important in the control of schistosomiasis, (Kaur and Arora, 2009).They also have anti-diarrheal, anti-septic, anti-viral, anti-fungal, anti-parasitic, andanti-irritant properties.Used in curbing hemorrhage, in wound healing, and improving vascular health by suppressing peptides that harden arteries, (Victor *etal*., 2005).

In this study, flavonoids were identified in all the four methanolic extracts and were abundant in *Azadirachta indica* leaves. Flavonoids exert their roles as chain-breaking antioxidants and preventing oxidation of low-density lipoprotein by macrophages and metal ions like copper, (Oduor *et al*., 2014). Flavonoids reduce the oxidative stressin humans (Awoyinka *et al*., 2007). They act as 'nature's biological modifiers', as anti-allergens, anti-inflammatory, and induces phase two enzymes that eliminate mutagens and carcinogens. They too work as anti-microbial by complexing extracellular and soluble proteins, and bacteria cell wall, (Awoyinka *et al*., 2007).

In this study, Saponins were identified in all the four methanolic extracts. Saponins boost respiratory system as an expectorant, and hence activity against a cough. This could justify the already traditionally established function of the plant in the treatment and

management of dry coughs. They also have anti-protozoa with cholesterol in the protozoal cell membranes causing cell lysis, (Oduor *et al*., 2016).

Saponins functions as a vaccine adjuvant, as anti-inflammatory, emetics, anti-viral, anti-fungal, insecticidal, molluscicidal, principal and as anti-bacterial by inhibiting colonization and boosting the immunity, (Miranda and Buhler, 2000). Saponins increase the blood flow in the coronary arteries, prevent platelet aggregation and decrease the consumption of oxygen by heart muscles, (Cheeke, 1998). They also have anti-edema, anti-tussive, purgative, anti hyper cholesterol, and hypotensive, cardiac depressant and immuno-regulatory properties, (Victor *et al*., 2005).

Terpenoids were identified in allthe extracts except in methanolic extracts of *Cassia siamea*in this study. Terpenoids exert their roles as anti-bacterial, anti-amoebic, anti-fungi, antiviral, anti-protozoal, anti-allergens, as immune boosters, and as anti-neoplastic. The mechanism of action is involve membrane disruption by these lipophiliccompounds, (Awoyinka *et al*., 2007). This may include perturbation of the lipid fraction of bacterial plasma membranes, altering membrane permeability hence causing leakage of intracellular materials, (Biradar *et al*., 2007).

In this study, the identification and characterization of the phytochemicals e.g., Saponins, flavonoids and terpenoids from methanolic extract of *Carissa edulis* root bark concurred with the findings of (Al-youssef, 2010) who upheld the presence of steroids, terpenes, tannins, and flavonoids. The identification and characterization of these phytochemicals also concurred with (Kokwaro, 2009), who established the presence of saponins from same plant species. The identification and characterization of phytochemical in this study concurred with, (Saidu*et al*., 2013) who used ethanol extracts of *Carissa edulis* root bark in rats and mice to establish its acute toxicity, and confirmed the presence of saponins, flavonoids and terpenoids while the steroids, cardiac glycosides, and tannins were not detected.

Saponins, steroids, flavonoids, terpenoids, tannins, and alkaloids from methanolic extract of *Azadirachta indica* leaves in this study concurred with several investigators. Phytochemical analysis and antimicrobial activity of *Azadirachta indica* A. Juss, (Vinoth *et al*., 2012) concurred with similar identification of alkaloids, steroids, saponins and flavonoids in acetone extracts of *Azadirachta indica* A. Juss. In this study, the identification and characterization of methanolic extracts of bioactive compounds e.g., alkaloids, steroids, saponins, tannins and terpenoids concurred with, (Harry-Asobara *et al*., 2014). The

identification and characterization of phytochemicals in the foliage of *Azadirachta indica* A. Juss, concurred also with, (Khan *et al*., 2007) findings.

The identification and characterization of phytochemicals of methanolic extracts of *Cassia siamea* Lam stem bark was inconsistent with, (Usha and Bopaiah, 2011) who used different organic solvents in carrying out phytochemical analysis. The presence of alkaloids, tannins, flavonoids, steroids and cardiac glycosides in ethanol extract, methanol extract, and ethyl acetate extract concurred with the discovery of this study. The presence of saponins in methanol extract and ethyl acetate extract of *Cassia siamea* Lam has further concurred with this study. In this study, terpenoids were not identified in methanolic extract of *Cassia siamea* stem bark. However, the identification of alkaloids, tannins, saponins and flavonoids from the methanolic extracts of *Cassia siamea* stem bark concurred with, (Mohammed *et al*., 2013; Alli Smith, 2009).

The identification and characterization of phytochemicals from *Harrisonia abyssinica* Oliv methanolic extract root bark of this study were same as in similar studies, (Ennocent and Magadula 2010) isolated steroids in methanolic extract of *Harrisonia abyssinica* species in Tanzania. Other findings of chemical groups e.g. saponins, tannins, flavonoids and terpenoids were not demonstrated in their work. Further more on the same plant species, (Balde *et al*., 2001) discovered terpenes in the phytochemistry tests. Similar findings have been reported, (Mungai *et al*., 2007) in their methanolic extracts of *Harrisonia abyssinica* root bark used as traditional antimalarial phytotherapy at the Kenyan coast.

In this study for *In vitro* anti-plasmodial activities, observations suggested that the active constituents in all the methanolic and aqueous extracts had anti-plasmodial activities on *Plasmodium falciparum* strains that were 3D7 and W2. Previous study on antiplasmodial activity of *Azadirachta indica* had been reported, (Ahmed *et al*., 1999) on selected Sudanese medicinal plants with emphasis on *Maytenus senegalensis* (Lam.). In this study, findings of *in vitro* anti-plasmodial activities of the methanolic and aqueous extracts are comparable with, (Mohammed *et al*., 2009) whose study was on assessment of antimalarial activity against *Plasmodium falciparum* and phytochemical screening of some Yemen medicinal plants. In this study, *in vitro* antiplasmodial activities of the four methanolic and aqueous extracts active constituents, (e.g., flavonoids, and terpenoids of methanolic extracts concurred with, (Dhar *et al*., 1998) in which extracts of *Azadirachta indica* were suggested to contain active constituents which might target specific metabolically active processes at the parasitic schizont stage. In a comparative study of acetone/water and aqueous extracts of *Azadirachta indica* leaves, they manifested inhibitory effect on a chloroquine-sensitive *Plasmodium*

falciparum at a concentration value of 20 µg/ml, (Iroka, 1993). In this study, antiplasmodial activities of methanolic extracts of *Azadirachta indica* showed IC_{50} values of 11.76 µM while aqueous extracts of *A. indica* gave IC_{50} values of 3.42 µM on sensitive strain of *Plasmodium falciparum*, (e.g., 3D7 chloroquine sensitive strain). However,earlier findings had shown that Azadirachtin of *A. indica* able to blockthe development of motile malaria gametes *in vitro* andraised the possibility of developing Azadirachtin-basedcompounds as antimalarial agents with transmission-blocking potential (Jones *et al*., (1994).

In vitro antiplasmodial activities in this study concurred with, (Kebenei *et al.*, 2011) whose findings on *Carissa edulis* showed IC_{50} value of 1.95 µg/ml. Although the methanolic and aqueous extracts of *Carissa edulis* showed IC_{50} values of 20.2 µM and 2.48 µM, for chloroquine sensitive strain and 16.12 µM and 12.7 µM for chloroquine resistant strain in this study, *Carissa edulis* showed good antiplasmodial activities on both on chloroquine sensitive resistant strain.

In vitro antiplasmodial activities of this study is in agreement with, (Al-Youssef and Hassan, 2014) who demonstrated different constituent's sesquiterpenes which were a class of compounds found in *Carissa edulis* pocessing antimicrobial, antimalarial, anticancer and anti-inflammatory effects. Although nine eudesmane -type sesquiterpenes were isolated from the methanolic extract of *Carissa edulis* root, these were carissone, cryptomeridiol, *β*-eudesmol, 6*α*-carissanol, 6*β*-carissanol, 2*α*-carissanol, 4-Epi-Aubergenone, and dehydrocarissone. The same plant extract a germacrane-type sesquiterpene, germacrenol, were also obtained, methanolic extract of *Carissa edulis* this study demonstrated only trace compounds e.g., steroids, saponins, tannins, flavonoids and terpenoids which have similar effects, (Sofowora, 1986; Achenbach, 1985).

I n this study, *in vitro* antiplasmodial activities of methanolic and aqueous extracts of *Cassia siamea* showed IC_{50} values of 3.08 µM and 5.19 µM for *P. falciparum* 3D7 strain and 11.35 µM and 12.85 µM for *P. falciparum* W2 strain. *In vitro* antiplasmodial activities of methanolic and aqueous extracts of *Cassia siamea* therefore concurred with (Bero *et al.*, 2009) whose identified 5-Acetonyl-7-hydroxy-2-methylchromone with IC_{50} value of 19.4 µM on *P. falciparum* 3D7 strain and Anhydrobarakol with IC_{50} value of 36.4 µM on *P. falciparum* 3D7 strain, (Pillay *et al*., 2007).

The *in vitro* antiplasmodial activities in the methanolic and aqueous extracts of *Harrisonia abyssinica* in this study showed IC_{50} values of 7.04µM and 4.03µM for *P. falciparum* 3D7 strain and 13.32 µM and 25.59 µM for *P. falciparum* W2 strain. The *in vitro* antiplasmodial

activities of *Harrisonia abyssinica* was in consistent with, (Kirira *et al*., 2006) who screened CQ-sensitive*Plasmodium falciparum* strain NF54 and CQ-resistant strain ENT30 against plant extracts from Meru and Kilifi County, Kenya and showed theIC_{50} value of 72.66 µg/ml for *Harrisonia abyssinica* root bark.

In this study, *in vitro* antiplasmodial activities of methanolic and aqueous extracts of the four medicinal plants were classified according to Muriithi and collaborator as follows; Methanolic extracts of *Carissa edulis* root bark had moderate activities on chloroquine sensitive strains of *Plasmodium falciparum* 3D7 strain and good activities on chloroquine resistant strains of *Plasmodium falciparum* W2 strain. Aqueous extracts have *C. edulis* root bark showed good antimalarial activities on both 3D7 and W2 strains of *P. falciparum*.

In vitro antiplasmodial activities of both methanolic and aqueous extracts of *Azadirachta indica* leaves, and *Cassia siamea* stem bark showed good antimalarial activities of *P. falciparum* 3D7 and W2 strains. Methanolic extracts of *Harrisonia abyssinica* root bark demonstrated good antimalarial activities on 3D7 and W2 strains of *P. falciparum* while aqueous extracts of *H. abyssinica* root bark showed good antimalarial activities on 3D7 and moderate antimalarial activities on *P. falciparum* W2 strain.

In this study, the cytotoxicity of the methanolic extracts was carried out using the brine shrimp test. Brine shrimp test was considered a rapid and inexpensive and bioassay for testing plant extracts lethal concentration which correlated with the cytotoxicity properties of the plants. Each of the methanolic extracts showed different mortality rates at different levels of concentrations. The percentage mortality increased with increase in the concentration of each methanolic extract. The variation in the brine shrimp cell dead was due to the difference in the amount and kind of cytotoxic substance found in the four methanolic extracts (e.g. steroids, saponins, glycosides, tannins, flavonoids, terpenoids, and alkaloids).The toxicity effect and the interactions of the four methanolic extracts were significant at $P < 0.05$. The present study concurred with (Mohammad *et al*., 2012) whose study was done on the phytochemical screening and cytotoxicity potential of ethanolic extracts of *Senna siamea* leaves and established the presence of tannins, steroids, and glycosides. This study concurred with, (Ayuko *et al*., 2009) whose study on *in vitro* antiplasmodial activity and toxicity assessment of plant extracts used in traditional malaria therapy in the Lake Victoria region. Their assessment of cytotoxicity using shrimps on methanolic stem bark and root bark extracts gave inactive and moderately active results respectively. The present study slightly differed with their work on *Carissa edulis* (Forssk) Vahl on stem bark analysis but gave similar outcome with root bark which showed presence of cytotoxic plant components of this

part of the plant. The current study concurred with, (Krishnan and Wong, 2015) in their work on *Azadirachta indica* acetone leaf extracts which gave high lethality of the brine shrimps. The organic solvent used for extraction was different from what was used in this study, but the outcome supported the presence of potent cytotoxic components found in *Azadirachta indica* leaves.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study identification and characterization of Steroids, Saponins, Glycosides, Tannins, Flavonoids, Terpenoids and Alkaloids from Siaya County were done. There was observable difference in the identification of the phytochemicals of the methanolic extracts.

The *in vitro* anti-plasmodial effects of the four medicinal plants were determined. There was significant difference on *Plasmodium falciparum* 3D7 and W2 strains determined. All the compounds showed antiplasmodial activities since they all demonstrated IC_{50}s. It was noted that when comparing methanolic and aqueous extracts on *Plasmodium falciparum* 3D7 and W2 strains, parasitaemia was observed to be highest in parasites treated with aqueous extracts. This led to significant difference of methanolic and aqueous extracts of the four medicinal plants from Siaya County.

The cytotoxicity effects of methanolic extracts from the four medicinal plants were low and safe. There was significant difference in the cytotoxicity of the methanolic extracts on *Artemia salina nauplii*. The study provided a scientific basis on the four medicinal plants used as antimalarial. It is possible that the extracts used together could provide synergistic effect in the control of resistant of *Plasmodium falciparum* W2 strains.

6.2 Recommendations

1. Seven phytochemicals (e.g., Steroids, Saponins, Glycosides, Tannins, Flavonoids, Terpenoids and Alkaloids) were identified and characterized in this study. However further studies are required to purify them for use against *Plasmodium falciparum* using advanced method like High Performance Liquid Chromatography.

2. Further research is recommended for *in vivo* anti-malarial activities since all the methanolic and aqueous extracts of the four medicinal plants demonstrated *in vitro* antiplasmodial activities at various $IC_{50s.}$

3. Further research is recommended to establish *in vivo* cytotoxicity profile since the methanolic extracts showed synergistic effect in control of resistant strains of *Plasmodium falciparum*.

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APPENDICES

Appendix 1**:** Two-way ANOVA.

Two-way ANOVA: 1000 µg/ml versus Extract, Effect

Source	DF	SS	MS	F	P
Extract	3	0.000	0.000	0.00	1.000
Effect	1	522.667	522.667	1045.33	0.000
Interaction	3	1.333	0.444	0.89	0.468
Error	16	8.000	0.500		
Total	23	532.000			

S = 0.7071 R-Sq = 98.50% R-Sq (adj) = 97.84%

Two-way ANOVA: 500 µg/ml versus Extract, Effect

Source	DF	SS	MS	F	P
Extract	3	0.000	0.000	0.00	1.000
Effect	1	294.000	294.000	441.00	0.000
Interaction	3	19.333	6.444	9.67	0.001
Error	16	10.667	0.667		
Total	23	324.000			

S = 0.8165 R-Sq = 96.71% R-Sq (adj) = 95.27%

Two-way ANOVA: 250 µg/ml versus Extract, Effect

Source	DF	SS	MS	F	P
Extract	3	0.000	0.0000	0.00	1.000
Effect	1	1.500	1.5000	0.69	0.418
Interaction	3	181.833	60.6111	27.97	0.000
Error	16	34.667	2.1667		
Total	23	218.000			

S = 1.472 R-Sq = 84.10% R-Sq (adj) = 77.14%

Two-way ANOVA: 125 µg/ml versus Extract, Effect

Source	DF	SS	MS	F	P
Extract	3	0.000	0.000	0.00	1.000

Source	DF	SS	MS	F	P
Effect	1	308.167	308.167	231.13	0.000
Interaction	3	88.500	29.500	22.13	0.000
Error	16	21.333	1.333		
Total	23	418.000			

S = 1.155 R-Sq = 94.90% R-Sq (adj) = 92.66%

Two-way ANOVA: 62.5 µg/ml versus Extract, Effect

Source	DF	SS	MS	F	P
Extract	3	0	0	*	*
Effect	1	600	600	*	*
Interaction	3	0	0	*	*
Error	16	0	0		
Total	23	600			

S = 0 R-Sq = 100.00% R-Sq(adj) = 100.00%

Two-way ANOVA: 31.25 µg/ml versus Extract, Effect

Source	DF	SS	MS	F	P
Extract	3	0	0	*	*
Effect	1	600	600	*	*
Interaction	3	0	0	*	*
Error	16	0	0		
Total	23	600			

S = 0 R-Sq = 100.00% R-Sq (adj) = 100.00%

Appendix 2

Table 10: Successive IC50 values for control drugs and compound against 3D7 strain of Plasmodium falciparum

3D7	CQ	MQ	C.a. *edulis*	C.a *siamea*	C.m. *siamea*	A. a. *indica*	H.a. *abysinica*	H.m. *abysinica*	A.m. *indica*	C. m. *edulis*
IC_{50}	2.587	4.01	253.8	6281	1062	654.7	9876	725.8	18120	12.99
	2.407	2.918	1013	1040	186	103	195.8	329.4	12727	218838
	5.037	3.057	137.3	6564	69036	3907	814353	5969	49513	56900
	1.452	3.616	48.18	58483	82546	13296	2009	942332	25428	103728
		4.861	12164	3907	17237	4.73	218838	2745	3.645	218838
	5.271	5.546	24.7	13296	2.109	2565	56900	2.092	2225968	56900
	5.553	3.032	60350	20	386359		103728	1.387	754555	103728
	4.738	3.249	6375	1665	5.084			7315	1.53	199905
	5.081	3.998	7.617	8762	69799			118011	1.37	23287
	4.919	3.461	2332		1.312			17708	3.05	37878
	5.422	3.828	56900					15637	3.51	19622
	5.088	4.424	103728					14515		
	2.868	0.8834						12477		
	6.426	2.08								
	4.307	2.76								
	6.822	14.71								
	3.888	2.605								

5.152	3.37
6.414	3.626
4.929	0.195
7.143	2.989
9.56	10.45
8.61	6.57
7.97	7.26
8.4	7.18
9.7	
8.8	
8.95	
7.5	

Key: 3D7 is chloroquine sensitive strain of Pf, CQ is chloroquine, MQ is mefloquine, C.a is aqueous extract of *C. edulis*, C.a is aqueous extract of *C. siamea*, C.m is methanolic extract of *C. siamea*, Aa is aqueous extract of *Azadirachta indica*, H.a is aqueous extract of *H. abyssinica*, H.m is methanolic extract of *H. abyssinica*, A.m is methanolic extract of *Azadirachta indica* and C.m is methanolic etract of *C. edulis*

Appendix 3:

Table 11: Successive IC_{50} values for control drugs and compounds against W2 strain of *Plasmodium falciparum*

W2	CQ	MQ	*C*.m. *edulis*	*C*.a. *edulis*	*A*.m. *indica*	*A*.a. *indica*	*C*.m.*siemea*	*C*.a.*siemea*	*H*.m.*abyssinica*	*H*.a.*abyssinica*
IC_{50}	2.24	1.37	47.5	48.89	79.39	62.59	5.038	26407	47763	34306
	4.824	4.518	7.22	1.14	21943	99.38	1.340	0.55	2338	3408
	2.25	1.4	14472	1.53	3.61	0.32	3.019	27673	13680	25800
	4.61	4.06	15462	11768	1.16	305536	4.664	0.79	8760	38828
	5.51	4.74	18684	25800	21824	528348	289008	69	12267	
	4.8	5.13	27014	38828	256538	14981	32233	1701	12061	
	5.99	4.38	1.89		1.07	99.86	35854	9.398	8585	
	5.4	10.47	26032		366202		97738	1.865	9075	
	5.62	5.18	40046		24354			2222	9960	
	8.08	5.31	19428					68717	8732	
	6.7	4.52						70373		
	22.4									
	5.36									
	6.7									
	8.99									
	11.38									
	6.82									
	7.02									
	5.64									

Key**:** W2 is mefloquine sensitive strain of *P.falciparum*, CQ is chloroquine, MQ is mefloquine, C.m is methanolic etract of *C. edulis*, C.a is aqueous extract of *C. edulis*, Ma is methanolic extract of *A. indica*, Aa is aqueous extract of *A. indica*,C.m is methanolic extract of *C. siamea*, C.a is aqueous extract of *C. siamea*, *H*.m is methanolic extract of *H. abyssinica* and H.a is aqueous extract of *H. abyssinica*.

Appendix 4

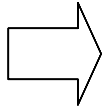
Table 12: Replication and percentage toxicity of each concentration of methanolic extracts of *C. edulis*, *A. indica,C. siamea* and *H. abyssinica*

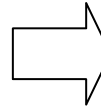
Concentrations µg/ml	1000 µg/ml		% tox	500 µg/ml		% tox	250 µg/ml		% tox	125 µg/ml		% tox	62.5 µg/ml		% tox	31.25 µg/ml		% tox	1%DMSO	
	D	L		D	L		D	L		D	L		D	L		D	L		D	L
A1	10	0	100	9	1	90	5	5	50	3	7	30	0	10	0	0	10	0	0	10
A2	9	1	90	8	2	80	7	3	70	0	10	0	0	10	0	0	10	0	0	10
A3	10	0	100	7	3	70	7	3	70	0	10	0	0	10	0	0	10	0	0	10
B1	10	0	100	10	0	100	9	1	90	0	10	0	0	10	0	0	10	0	0	10
B2	10	0	100	10	0	100	6	4	60	0	10	0	0	10	0	0	10	0	0	10
B3	10	0	100	10	0	100	5	5	50	0	10	0	0	10	0	0	10	0	0	10
C1	8	2	80	7	3	70	0	10	0	0	10	0	0	10	0	0	10	0	0	10
C2	10	0	100	9	1	90	0	10	0	0	10	0	0	10	0	0	10	0	0	10
C3	10	0	100	7	3	70	0	10	0	0	10	0	0	10	0	0	10	0	0	10
D1	9	1	90	8	2	80	4	6	40	3	7	30	0	10	0	0	10	0	0	10
D2	10	0	100	9	1	90	7	3	70	5	5	50	0	10	0	0	10	0	0	10
D3	10	0	100	8	2	80	7	3	70	6	4	60	0	10	0	0	10	0	0	10

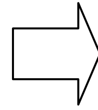
Key:A1, A2, and A3 are replicates of *C. edulis*, B1, B2, and B3 are replicates of *A. indica*, C1, C2, and C3 are replicates of *C. siamea*, D1, D2, and D3 are replicates of *H. abyssinica*, % tox = percentage toxicity, 1% DMSO = one percent dimethyl sulfoxide, D = Dead and L = Life.concentration (1000, 500, 250, 125, 62.5, 31.25 µg/ml) of methanolic extracts of *C.edulis, A. indica, C.siamea*and *H.* abyssinica.

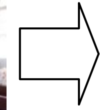
Appendix: 5a

Determination of phytochemicals of methanolic extracts of *Carissa edulis, Azadirachta indica, Cassia siamea* and *Harrisoniaabyssinica*









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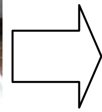
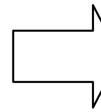
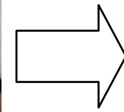
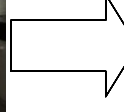
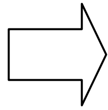








6 **7** **8** **9** **10**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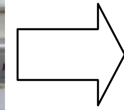

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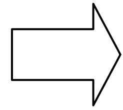
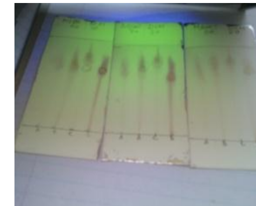
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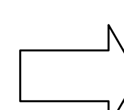
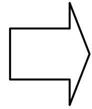
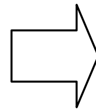


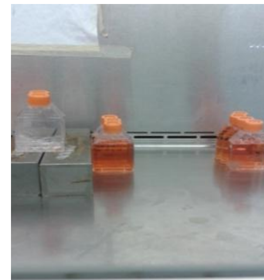
Appendix: 5b

Determination of anti-plasmodial effect of methanolic andaqueous extracts of *Carissa edulis, Azadirachta indica, Cassia siame*a and*Harrisonia abyssinica* on CQ - R and CQ - S, and MQ - R and MQ - S *Plasmodium falciparum* strains.



Revival *P. falciparum* 1





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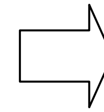

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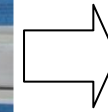

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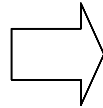

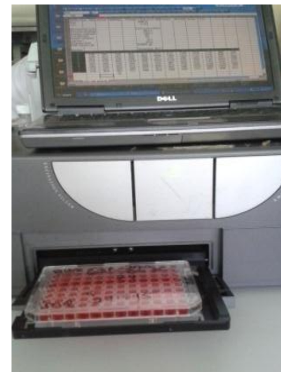

5

Appendix 5c

Determination of cytotoxicity of *Carissa edulis*, *Azadirachta indica*, *Cassia siamea* and *Harrisonia abyssinica* methanolic extracts on brine Shrimp larvae (*Artemia salina nauplii*).