

In Vitro* Antiplasmodial Activities of Crude Extracts of *Carissa edulis*, *Azadirachta indica*, *Cassia siamea* and *Harrisonia abyssinica* against *Plasmodium falciparum

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Abstract

The emerging resistance of *Plasmodium falciparum* to chloroquine and sulfadoxine pyrimethamine drugs in Kenya has necessitated the need to look for new more effective antimalarial drugs. This study investigated the *in vitro* antiplasmodial activities of methanolic and aqueous crude extracts of *Carissa edulis*, *Azadirachta indica*, *Cassia siamea* and *Harrisonia abyssinica* on *Plasmodium falciparum* strains. Drug assays were conducted using SYBR Green 1 dye. Two Reference *Plasmodium falciparum* strains, 3D7 and W2 were assayed against two anti-malarial conventional drugs and crude extracts of *Carissa edulis*, *Azadirachta indica*, *Cassia siamea* and *Harrisonia abyssinica* to generate 50% inhibitory concentrations of chloroquine, mefloquine, methanolic and aqueous extracts. These extracts have potential for antimalarial activities that can be used to develop pure compounds for prospective antimalarial molecules for therapeutic uses. Methanolic and aqueous extracts of *Carissa edulis*, *Azadirachta indica*, *Cassia siamea* and *Harrisonia abyssinica* demonstrated *in vitro* antiplasmodial effect on the two *Plasmodium falciparum* strains. These findings support the hypothesis that these plants have antiplasmodial activities against *Plasmodium falciparum* strains through the active phytochemicals found in them. The extracts from these plants therefore have the potential to be harnessed for anti-malarial drug development.

Key words: Antiplasmodial, *Plasmodium falciparum*, Phytochemicals, Methanolic extracts, Aqueous extracts

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Introduction

Malaria is a common tropical disease affecting many people. It is caused by a protozoan parasite of the genus *Plasmodium*. The parasite is transmitted by *Anopheles* mosquito as a definitive host

from one human - being to another through bite and introduction of the parasite into the human host blood stream. Five species of mosquito - borne *Plasmodium* parasites infect humans; these include *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* and *Plasmodium falciparum*. Among these, *Plasmodium falciparum* causes the most virulent form of malaria, and the highest mortality rates and morbidity in children under the age of five years, pregnant women and old people. More than 2.4 billion people from over 90 countries worldwide have the disease burden experienced in children with the biggest percentage from sub-Saharan African (WHO, 2012). The disease caused by *Plasmodium falciparum*, if not properly treated and managed in time, may result into the parasite developing resistance to the antimalarial drugs (Greenwood *et al.*, 2008). From West Africa and Southeast Asia, *P. falciparum* revealed an unexpected cluster of clonally propagated parasite subpopulations resistant to artemisinin, which is a key antimalarial drug (Carlton *et al.*, 2008).

In Kenya, malaria causes significant morbidity and mortality with a number of parasite resistant to drug cases being reported, especially the resistance to chloroquine diphosphate (CQ) and sulfadoxine pyrimethamine (Co EM *et al.*, 2009). The World Health Organization estimates that 80% of the world's population depends on medicinal plants for their primary health care (GuribFakim *et al.*, 2007; Mothana *et al.*, 2008; Gupta *et al.*, 2010). In this regard, the use of traditional medicine has been explored globally as folklore medicine among people in developing countries in their health care systems as an alternative where conventional medicine is less available (Gupta *et al.*, 2010; Rates, 2001). Natural products are important source of new antimicrobial agents which are in the form of secondary metabolites (Cowan, 1999). The bioactivity of a number of plants have been investigated by many researchers worldwide giving positive and promising results (Cowan, 1999; Hannan *et al.*, 2011). Natural products either as pure compounds or as standardized plants extracts, provides unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Parekh and Chanda, 2007). The aim of this study was to investigate the *in vitro* antiplasmodial activities of methanolic and aqueous crude extracts of the four plants *Carissa edulis*, *Azadirachta indica*, *Cassia siamea* and *Harrisonia abyssinica* commonly used by herbalists for treatment of various ailments.

Materials and Methods

Plants' roots, stems and leaves from which the crude extracts were made were collected from Masumbi village, in Siaya County, Kenya from January to February 2015 and processed as described by Oduor *et al.*, (2016).

Preparation of *Plasmodium falciparum* Culture Medium

The *in vitro* *P. 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 960ml 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 0.2µm filter unit, and labelled with the expiry date, (Trager and Jensen, 1976).

Activation of *Plasmodium falciparum* Strains from Liquid Nitrogen

Reference *P. falciparum* strains in liquid nitrogen were stored for future use. For the revival, they were located in the storage and pulled out for processing. They were transferred into bio-safety cabinet and thawed over water bath at 37°C. The amount thawed was transferred into 15ml centrifuge tube, and the volume marked. The amount thawed was picked and divided by 5. 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 (CO₂), 90% nitrogen (N₂) and 5% oxygen (O₂) and then incubated at 37°C under moisture condition, (Amaratunga, *et al.*, 2013; Akala *et al.*, 2011).

Maintaining *Plasmodium falciparum* Strains (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 0.5ml 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 *P. falciparum* parasites. Caps were then removed from the flask containing medium, and 4.5ml of medium was aspirated into the serological pipette and expelled into the 25cc culture flasks. The red cells were resuspended and the bottles gently agitated in a circular motion. The flasks were flushed with 5% CO₂, 90% N₂ and 5% O₂ gas mixture and then placed at 37°C in an incubator under moisture condition.

***Plasmodium falciparum* Parasitemia in Thin Blood Smear**

A volume of 10µl 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 laminarcabinet. The slides were stained using 1 in 10 dilutions of Giemsa stain for 20 minutes. Three fields were randomly selected and observed using ×100 objective lens and on each field count, the number of

red blood cells and the number of parasites were counted upto 2,000. Parasitaemia was calculated as the number of parasites divided by the total number of red blood cells.

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 5 ml, 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 510 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% CO₂, 90% N₂ and 5% O₂. The new cultures were again mixed in a circular motion and placed horizontally in the 37°C incubator.

Preparation of Standard Drugs (Chloroquine and Mefloquine)

This was done under a bio-safety cabinet. Stock solutions of 5mg/ml were prepared in 5ml of 100 % dimethyl sulfoxide (DMSO) for chloroquine (CQ) and mefloquine (MQ) respectively. $CQ = 5 \text{ mg/ml} \times 1,000,000 = 5,000,000 \text{ ng/ml}$. Starting concentration on the plate was reduced to 2000 ng/ml. Therefore $5,000,000 \div 2,000 = (1: 2,500) \times 9 = 9\mu\text{l}$ in 2,500 μl , hence $4 \times (9 \times 2,500) = 36\mu\text{l}$ in 10,000 μl divided by 1,000 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. $5,000,000 \div 500 = 10,000$ hence $9 \times (1\mu\text{l}$ in 10 ml) = 9 μl in 10 ml. About 300 μl of MQ was picked and transferred into the 2nd well of the 1st column of the 384 microtiter plate. Further dilutions were done in complete medium with serum (150 μl) to reach the desired starting concentration of 2000ng/ml and 500ng/ml for CQ and MQ respectively. A serial 2-fold dilution followed to generate 10 concentrations for IC₅₀ testing. Concentrations range from the highest to lowest were 2000ng/ml to 1.977 and 500ng/ml to 0.488ng/ml for CQ and MQ respectively.

Preparation of Test Drugs (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. Starting concentration 10,000,000 ng/ml was reduced to 50,000ng/ml. 10,000,000ng/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.

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, up to 8 of the 384 microtiter plates. Complete RPMI 1640 medium, 150 μl was added to a 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.

Preparation of Secondary Culture (Daughter) Plates

Daughter plates were placed in the same orientation with the mother plate. Volume of 12.5µl 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.

In vitro Antiplasmodial Activity Assay

Antiplasmodial activities of methanolic and aqueous crude extracts and reference drugs were assayed against chloroquine-sensitive, (3D7) and chloroquine-resistant Indochina 1 (W2) *P. falciparum* strains using a nonradioactive assay technique. The technique uses deoxyribonucleic acid (DNA) dye that accurately depicts *in vitro* parasite replication. This fluorochrome is known as SYBR Green 1, anon-radioactive DNA dye (Wanyama *et al.*, 2011). Laboratory cloned parasites were obtained from liquid nitrogen, thawed at 37°C and cultured as described by Akala *et al.*, (2011) to establish replication robustness of 3 - 6% parasitemia. Briefly stock solutions 5mg/ml were prepared in 5 ml of 100% dimethyl sulfoxide (DMSO) for chloroquine (CQ) and mefloquine (MQ). Concurrently a total of 45 mg of methanolic and aqueous extracts were weighed using analytical balance, (Denver Instrument- XL 3100D), and dissolved in 100% DMSO and vortexed in biosafety cabinet using Barnstead/Thermolyne 2555 Bonlevard, USA. Two-fold serial dilutions of the reference drugs chloroquine 2000ng/ml - 1.977ng/ml, mefloquine 500ng/ml - 0.488ng/ml and test samples, 50000ng/ml - 48.83ng/ml were prepared in a 384 micro culture plate. The culture adapted *Plasmodium falciparum* were reconstituted to 1% parasitemia and added to the micro culture plates containing dose range of the reference drugs and test samples and incubated in a gas mixture of 5% CO₂, 5% O₂ and 90% N₂ at 37°C.

The assay was terminated after 72 h and frozen at -80 °C for 24 h. Lysis buffer containing SYBR Green1 was added to the plates and gently mixed using the Beckman Coulter Biomek 2000 automated Laboratory Workstation (Beckman Coulter, Inc. Fullerton, CA). The plates were incubated for 15 minutes in the dark at room temperature. Parasites replication inhibition was quantified and the IC₅₀ for each drug and test samples calculated by an equation generating a sigmoidal concentration-response curve (variable slope), with log transformed drugs and test samples concentrations on the Xaxis and relative fluorescence units (RFUs) on the Y axis (Graph Prism for Windows' version 5.0; Graphpad Software, Inc., San, Diego, CA).

Results and Discussions

Chloroquine, mefloquine, methanolic and aqueous extracts of the four plants was assayed against *P. falciparum* 3D7 and W2 strains and the comparison of the mean IC₅₀s against relative fluorescence units (RFUs) demonstrated.

The *in vitro* anti-plasmodial activities showed that the active constituents in all the methanolic and aqueous extracts had antiplasmodial activities against *P. falciparum* 3D7 and W2 strains. The following extracts showed similar 50 % Inhibitory concentration (IC₅₀) to that of chloroquine (CQ) assayed against 3D7 strain: methanolic extracts of *C. siamea* stem bark, *H. abyssinica* root bark and aqueous extract of *H. abyssinica* root bark (see Figure 1).

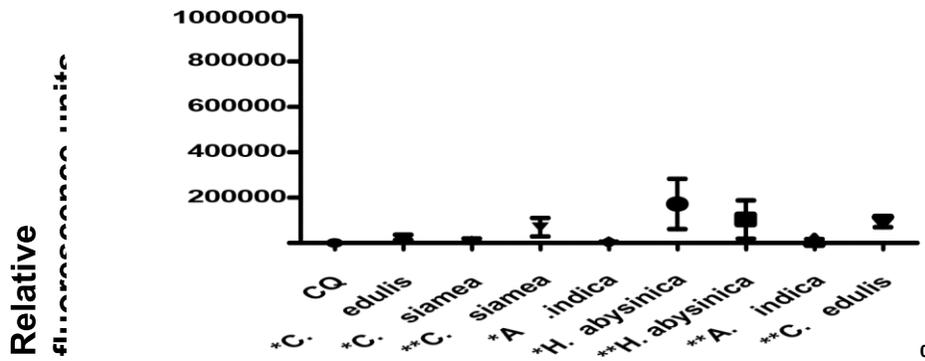


Figure 1: IC_{50} plots for chloroquine, methanolic and aqueous extracts against *Plasmodium falciparum* 3D7 strain RFUs. CQ is chloroquine and **methanolic and *aqueous extracts from given the four plants

Similar IC_{50} to that of CQ assayed against W2 strain was observed in methanolic extracts of *A. indica* leaves, *Cassia siamea* stem bark and aqueous extract of *A. indica* leaves (see Figure 2).

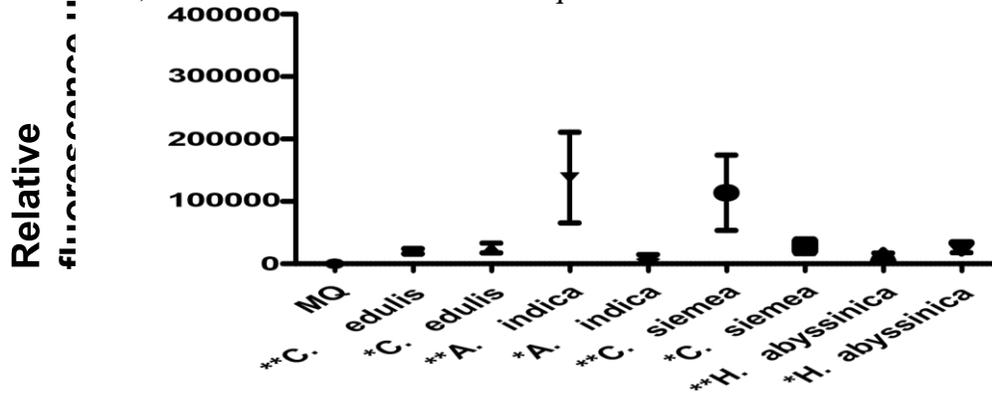


Figure 2: IC_{50} plots for mefloquine, methanolic (**) and aqueous (*) extracts from given plants against *Plasmodium falciparum* W2 strain RFUs

In the current study, methanolic extracts of *C. siamea* stem bark, *A. indica* leaves, *C. edulis* root bark, aqueous extracts of *H. abyssinica* root bark and *C. edulis* root bark showed similar IC_{50} to that of MQ assayed against 3D7 strain (see Figure 3).

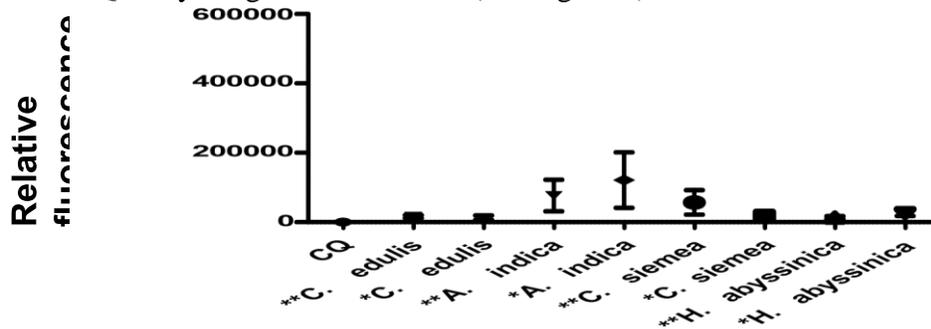


Figure 3: IC_{50} plots for chloroquine, methanolic (**) and aqueous (*) extracts from given plants against *Plasmodium falciparum* W2 strain.

Similarly, methanolic extracts of *A. indica* leaves, *C. siamea* stem bark and aqueous extract *A. indica* leaves showed IC_{50} similar to that of MQ assayed against W2 strain (see Figure 4).

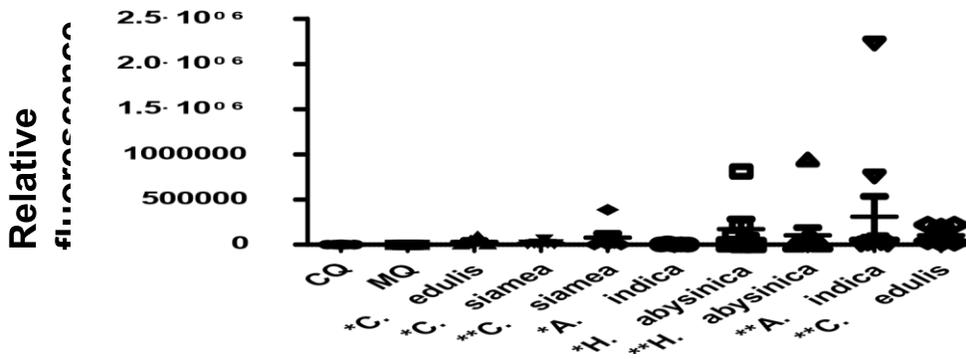


Figure 4: IC₅₀ Plots for chloroquine and mefloquine, methanolic(**) and aqueous (*) extracts from the given plants against *Plasmodium falciparum* 3D7 strain RFUs.

In this study methanolic extracts of *H. abyssinica* root bark, *A. indica* leaves and aqueous extract of *H. abyssinica* root bark showed similar IC₅₀ to CQ and MQ assayed against 3D7 (see Figure 5).

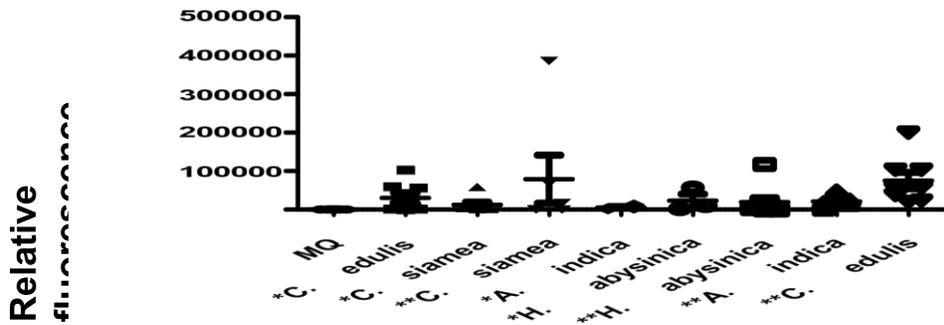


Figure 5: IC₅₀ plots for mefloquine, methanolic (**) and aqueous (*) extracts from given plants against *Plasmodium falciparum* 3D7 strain RFUs.

This was similarly observed with methanolic extracts of *C. siamea* stem bark; *A. indica* and aqueous extract of *A. indica* leaves assayed against W2 strain (see Figure 6).

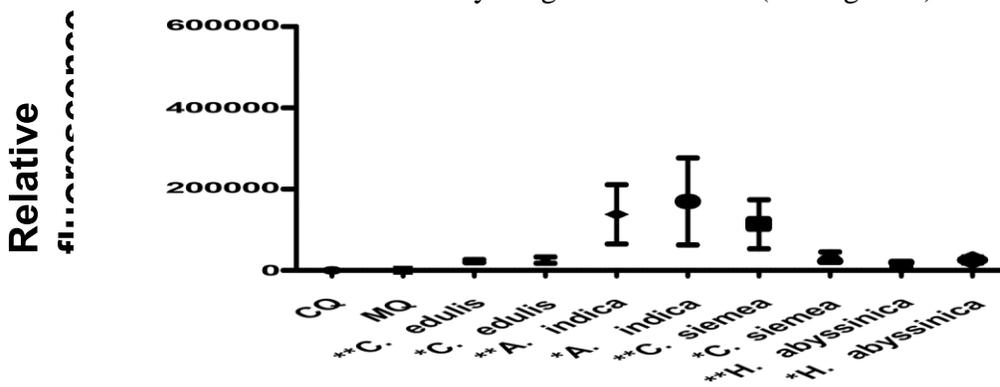


Figure 6: IC₅₀ plots for chloroquine, mefloquine, methanolic and aqueous extracts against *Plasmodium falciparum* W2 RFUs.

Previous study on antiplasmodial activity of *A. 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* antiplasmodial activities of the methanolic and aqueous extracts are comparable with those of Mohammed *et al.*, 2009 whose study was on assessment of antimalarial activity against *P. 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 *A. 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 *A. indica* leaves; they manifested inhibitory effect on a chloroquine-sensitive *P. falciparum* at a concentration value of 20µg/ml (Iroka, 1993). In this study, antiplasmodial activities of methanolic extracts of *A. indica* showed IC₅₀ values of 11.76µM while aqueous extracts of *A. indica* gave IC₅₀ values of 3.42µM on sensitive strain of *P. falciparum*, (e.g., 3D7 chloroquine sensitive strain). However, earlier findings had shown that Azadirachtin in *A. indica* was able to block the development of motile malaria gametes *in vitro* and raised the possibility of developing Azadirachtin-based compounds as antimalarial agents with transmissionblocking potential (Jones *et al.*, 1994).

In vitro antiplasmodial activities in this study concurred with those of Kebenei *et al.*, (2011) whose findings on *C.edulis* showed IC₅₀ value of 1.95µg/ml. Although the methanolic and aqueous extracts of *C.edulis* showed IC₅₀ values of 20.2 µM and 2.48 µM, for chloroquine sensitive strain was 16.12 µM and 12.7 µM for chloroquine resistant strain in this study, *C. edulis* showed good antiplasmodial activities on both chloroquine sensitive resistant strain.

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

In the study, *in vitro* antiplasmodial activities of methanolic and aqueous extracts of *C. siamea* showed IC₅₀ 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-Acetyl-7-hydroxy-2-methylchromone with IC₅₀ value of 19.4µM on *P. falciparum* 3D7 strain and Anhydrobarakol with IC₅₀ value of 36.4µM on *P. falciparum* 3D7 strain, (Pillay *et al.*, 2007). The *in vitro* antiplasmodial activities in the methanolic and aqueous extracts of *H. abyssinica* in this study showed IC₅₀ values of 7.04µM and 4.03µM for *P. falciparum* 3D7 strain and 13.32µM and 25.59µM for *P. falciparum* W2 strain. The *in vitro* antiplasmodial activities of *H. abyssinica* was consistent with Kirira *et al.*, (2006) who screened

CQ-sensitive *P. falciparum* strain NF54 and CQ-resistant strain ENT30 against plant extracts from Meru and Kilifi County, Kenya and showed the IC₅₀ value of 72.66 µg/ml for *H. abyssinica* root bark.

In that 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 *P. falciparum* 3D7 strain and good activities on chloroquine resistant strains of *P. falciparum* W2 strain. Aqueous extracts of *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 *A. indica* leaves, and *C. siamea* stem bark showed good antimalarial activities of *P. falciparum* 3D7 and W2 strains. Methanolic extracts of *H. 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.

Conclusions and Recommendations

The *in vitro* anti-plasmodial activities of the four medicinal plants were determined. This study supports that *C. edulis*, *A.indica*, *C. siamea* and *H. abyssinica* have antiplasmodial activities to different *P. falciparum* strains. The antiplasmodial activities were associated with the variety of the active phytochemicals that are found in these plants. These plants have the potential to be harnessed for further study in anti-malarial drug discovery and especially the ones that have shown IC₅₀ similar to chloroquine and mefloquine.

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