Research in Pharmaceutical Biotechnology Vol. 2(4), pp. 40-50, August 2010
Available online at http://www.academicjournals.org/rpb
ISSN 2141-2324 ©2010 Academic Journals

Full Length Research Paper

In vitro antiplasmodial and cyclin- dependent protein kinase (pfmrk) inhibitory activities of selected flavonoids in combination with chloroquine (CQ) and artemisinin

H. M. Akala[1], C. N. Waters[1], A. Yenesew[2], C. Wanjala[3] and T. Ayuko Akenga[4]*

[1]Kenya Medical Research Institute, Walter-Reed Laboratories, P. O. Box 54840 - 00100 G. P.O, Nairobi, Kenya.
[2]Department of Chemistry, University of Nairobi, P. O. Box 30197 - 00100 G. P. O. Nairobi, Kenya.
[3]Department of Physical Sciences South Eastern University College (SEUCO) P. O. Box 170-90200, Kitui, Kenya.
[4]Office of the Deputy Principal (Academic Affairs), Bondo University College (BUC) P. O. Box 210-40601, Bondo, Kenya.

Accepted 2 February, 2010

In this study, we report *in vitro* chloroquine (CQ) and artemisinin combination studies of eight flavonoids against *Plasmodium falciparum* strains. The flavonoids were previously isolated from *Erythrina sp.* (Family Leguminosae) growing in Kenya. Synergism was observed for chloroquine/ Sigmoidin E (5) combination at ratios of 5:1 and 3:1 respectively, while other chloroquine/flavonoids combinations showed variable response; either additive or even antagonistic effects. The artemisinin/abyssynone IV (1) combination also showed synergistic interaction at a ratio of 5:1. Further investigations revealed that Abyssinone IV (1) and Abyssinone V (2) were effective inhibitors of plasmodial growth and differentiation regulatory enzyme, pfmrk.

Key words: Combination studies, CQ, artemisinin, flavonoids, sigmoid E, synergism, antagonism, pfmrk.

INTRODUCTION

The theory underlying combination drug management of tuberculosis, leprosy, and HIV infection is well known and is now generally accepted for malaria. Combination of drugs greatly lowers the probability of emergence of resistance (Nicholas, 2004; Bell, 2005). The change from monotherapy to combination therapy for malaria was effected and enforced before the year 2000 in most malaria endemic countries by government policies. Several drug combinations for treatment of malaria are now in use. They include pyronaridine/artemisinin; chlorproguanil/dapsone; chlorproguanil/dapsone/artesunate and artemether /lumefantrine (Coartem®) combinations.

However, the fast emergence of resistance to the existing cheaply available combinations, like sulfadoxine/ pyrimethamine, is a major threat to the future of malaria treatment and control efforts (Krishna et al., 2006; Nzila et al., 2000). Consequently, this medication has been replaced with the more effective but costly Coartem® (Mutabingwa et al., 2001). Furthermore, there is an overall lack of chemical diversity among the commercially available antimalarial drug compounds except for artemisinin (Macreadie et al., 2000), a problem that is further compounded by the fact that resistance to artemisinin has been reported in various regions (Taylor and White, 2004; Clark, 1996).

Recent studies have shown *in-vitro* antiplasmodial activities of extracts from plants traditionally used as antimalarials (Robert, 2003; Muregi et al., 2004; Brandao et al., 1997; Francois et al., 1996; Freiburghaus et al., 1996). Some of the most interesting results were from a plant called *Erythrina* sp. (Family Leguminosae). The plants in this family are widely used in traditional medicine for treatment of malaria and microbial infections (Kokwaro, 1993). Antiplasmodial activities of flavonoids from the roots and stem bark of these species have been reported (Yenesew et al., 2005; 2004; 2003a, b and c).

Cyclin-dependent protein kinases (CDKs) are attractive

*Corresponding author. E-mail: tezakenga@yahoo.co.uk, takenga@bondo-uni.ac.ke.

targets for drug discovery. Recently efforts have led to the identification of novel CDK selective inhibitors in the development of treatments for cancers, neurological disorders, and infectious diseases and CDKs have now become the focus of rational drug design programs for the development of new antimalarial agents. CDKs are valid drug targets as they function as essential regulators of cell growth and differentiation.

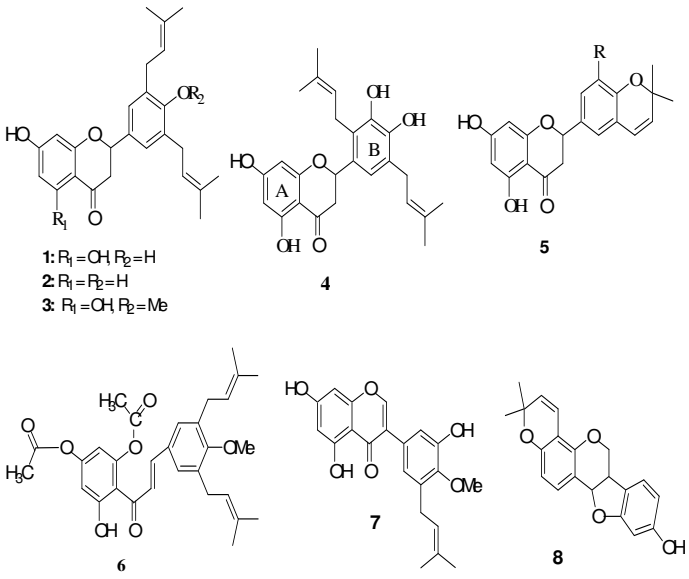
To date, several CDKs, pfmrk and PfPK5, have been characterized from the genome of the malaria-causing protozoan *Plasmodium falciparum* (Canduri et al., 2005; Keenan et al., 2006). Many of the parasitic protein kinases display profound structural and functional divergences from their host counterparts and pfmrk and PfPK5, most likely play an essential role in cell cycle control and differentiation in *P. falciparum*. This, therefore, makes them an attractive target for antimalarial drug development (Geyer et al., 2009). Various 1,3-diaryl-2-propenones (chalcone derivatives) which selectivity inhibit pfmrk in the low micromolar range (over PfPK5) have been reported. It is believed that kinase inhibition could be an additional mechanism of antimalarial activity for this class of compounds.

In this study, potencies of chloroquine (CQ)/flavonoid and artemisinin/flavonoid combinations were evaluated against CQ sensitive (D6) and CQ resistant (W2) strains of *P. falciparum*. The anti-*P. falciparum,* M015 Related Kinase (*pf*mrk), of selected flavonoids has been evaluated, in an attempt to contribute to the discovery of new anti-malarial drugs.

MATERIALS AND METHODS

Flavonoids assayed

Flavonoids 1 - 8 were assayed in this study. These compounds were from the library of compounds isolated, characterized and reported by Yenesew et al., in their studies of 2003, 2004 and 2005.



Antiplasmodial activity assay

Antiplasmodial activity test was based on the procedure previously described by Desjardins et al. (1979). *P. falciparum* parasites were maintained by standard culture method (Chulay et al., 1983; Trager and Jansen, 1997). A two-fold serial drug dilution on 96-well plates was done using the Berkmann 1000 TM automated laboratory workstation with 50 µg/ml as starting concentration for all test compounds. The reference drugs had varied preset starting dose. Out of the eight rows on each plate, two were reference antimalarials drugs - chloroquine and quinine- while six were the different test samples.

For combination assays, a 96-well plate had first rows containing the particular reference drug, and row two, the test sample. The remaining six rows contained reference drug to sample drug concentration ratios of 1:1, 3:1, 1:3, 4:1, 1:4 and 5:1 respectively. The proportions were volume/volume parts of the combining components at the preset working doses such that reference drugs were at either 1000 mg/ml for chloroquine sulphate or 100 mg/ml for artemisinin and 50 µg /ml for the sample drugs. For the assay, 25 µl aliquots of the drug were pipetted into each of the microtitre wells on the plates. *P. falciparum* parasites were kept in continuous culture to adapt and attain a parasitemia of 3 - 6% trophozoite stages as proof to successful adaptation to *in-vitro* growth.

Aliquots of 200 µl of a 0.9% (v/v) suspension of parasitized erythrocytes in culture medium were added to all test wells except the three negative control wells. The plates were incubated at 37°C in a gas mixture of 3% CO_2–5% O_2–92% N_2. After 24 h, each well was pulsed with 25 µl of culture medium containing 0.5 µCi of [^{3}H] hypoxanthine and incubated for a further 18 h.

The contents of each well were harvested onto glass fiber filters, washed thoroughly with distilled water, dried, and the radioactivity (in counts per minute) measured by liquid scintillation counting. These tests were carried out in triplicates. The concentration causing 50% inhibition of radioisotope incorporation (IC_{50}) was determined by interpolation as described by Desjardins et al. (1979).

The highest concentrations of drugs were placed in the first column and subjected to a two-fold serial dilution across the row. Each plate had two control drugs, that is, the first two drugs (row A-chloroquine sulphate, and row B- quinine sulphate).

Data analysis

Oracle database software (Data Aspects Corporation, California USA, 1996) was employed in analysis of data to give 50% inhibitory concentration (IC_{50}). It is based on the inhibition of uptake of a radioactive nucleic precursor (hypoxanthine) by parasites as a measure of antiplasmodial activity. This program compares the individual counts to the background and the positive control, and plots a dose response curve (Frederich et al., 1999).
IC_{50} was calculated using Equation 1:

$$\% \text{ inhibition} = 100 - \frac{(\text{cpm test} - \text{mean cpm UC})}{(\text{mean cpm infected cells} - \text{mean cpm UC})} \times 100 \quad (1)$$

where cpm = counts per minute, UC = uninfected cells

Exclusion was considered for all assays that showed: Bacterial contaminations, IC_{50} incorrectly aligned (failure to converge), low counts in the control wells, and inability to fit a logistic dose-response equation as shown by the analysis program. For

Table 1. *In vitro* IC_{50} values of flavonoids isolated from *Erythrina sp.* against W2 and D6 strains of *P. falciparum.*

Category of activity	Compound	IC_{50} (µMl)	
		D6	W2
A	Abyssinone IV (1)	5.4 ± 1.5	5.9 ± 1.8
	Abyssinone V (2)	2.4 ± 0.2	3.6 ± 0.2
	Sigmoidin E (5)	9.1 ± 2.3	11.8 ± 2.5
	5′-Prenylpratensein (7)	6.3 ± 0.3	8.7 ± 1.5
	Shinpterocarpin (8)	6.6 ± 1.2	8.3 ± 1.1
	Sigmoidin A (4)	5.8 ± 0.6	5.9 ± 1.1
B	Abyssinone V-4′ -methyl ether (3)	11.3 ± 2.1	11.1 ± 2.4
	Abyssinone 4′ -methyl ether diacetate (6)	12.3 ± 3.1	12.4 ± 0.1
	Reference drugs		
Chloroquine		0.008 ± 0.0	0.075 ± 0.0
Quinine		0.050 ± 0.02	0.28 ± 0.02

combination assays, it gave 50% fractional inhibition concentration (FIC_{50}) plot for every set of combination depicting synergistic, additive and antagonistic effects. Scatter plots were generated from the FIC_{50} to explain the potency of a given combination by putting all the triplicate assays on one plane. Proguanil/artovaquone combination was used as the positive control.

Pfmrk kinase inhibition assays

Expression and purification of pfmrk was performed as described by Li et al., (1996). The kinase assays were performed according to the procedure of Roch et al., (2000). A standard reaction (30 µl) contained 25 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 2 mM $MnCl_2$ 15 µM ATP/0.05 µCi of [γ -^{32}P] ATP, and 5 µg of histone H1 (Life Technologies, Inc.). Reactions were initiated by the addition of 0.5 µg each of the recombinant protein kinase and cyclin H as a partner. Both proteins were allowed to form a complex at 30°C for 30 min in kinase assay buffer.

The respective flavonoids were then added and incubated at 30°C for 30 min; the negative controls were reaction mixtures containing only the relevant concentration of solvent. The reaction was stopped by the addition of Laemmli buffer. A fixed volume (25 µl) of each reaction was then spotted onto a small piece of Whatman P81 phosphocellulose paper. The paper was washed five times in 1% orthophosphoric acid, and the amount of acid-precipitable radiolabel incorporated in histone H1 was quantified by scintillation counting (Roch et al., 2000). From the differential counts, 50% inhibitions (IC_{50}) were established.

RESULTS AND DISCUSSION

Flavonoids 1-8 were selected based on their reported antiplasmodial activity, which is either in category A or category B (Yenesew et al., 2005; 2004a; 2003a, b and c). These activities were confirmed during this study (Table 1). It was necessary to re-evaluate the antiplasmodial activities of the above compounds. This is because these compounds had been stored for a long time and their activity could have been affected.

All the flavonoids tested showed activities within IC_{50} of 10 - 20 µM. Growth inhibition of the chloroquine-sensitive (D6) strain by chalcones was better than that of the chloroquine-resistant (W2) strain.

Chalcone, abyssinone V-4′ -methyl ether (6) was prepared by acetylation of abyssinone V-4′ -methyl ether (3) under reflux condition in an attempt to prepare abyssinone V-4′ -methyl ether diacetate. However, the product formed a chalcone 6 where ring C has opened. Interestingly, the chalcone showed comparable antiplasmodial activity with that of the flavanone 3.

From this study, the tested compounds can be identified as lead antimalarial structures since they had activities in the same range as the lead antimalarial compound licochalcone A (Ziegler et al., 2004). Structure–activity relationship studies on the antimalarial activity of chalcones have emphasized the importance of ring B for activity (Ziegler et al., 2004). In particular, parasubstitution of ring B with oxygen and non-bulky substituents like hydrogen has been reported to be desirable (Liu et al., 2003). All tested chalcones had an oxygen group in this position and showed antiplasmodial activity in the range of category B. Notably, antiplasmodial activity among these chalcones was comparable in spite of the changes in size, type and position of substituents on ring A.

Combination studies

The potency of flavonoids in combination with either artemisinin or CQ was evaluated in assay against two strains of *P. falciparum.* The flavonoids 1 - 8 being natural products, were in short supply. The number of assays carried out was limited by the availability of these compounds.

Each of flavonoids 1 and 3 was used in combination

Table 2. FIC_{50} for atovaquone/Proguanil combinations activity on D6 strain of *P. falciparum.*

Combining ratio	Mean sum FIC_{50}s ±SD
1:1	0.9 ± 0.1
3:1	0.9 ± 0.1
1:3	1.2 ± 0.2
4:1	0.9 ± 0.1
1:4	0.7 ± 0.2
5:1	1.4 ± 0.2

Number of replicates, n = 3, D6 = CQ sensitive strain of *P. falciparum*.

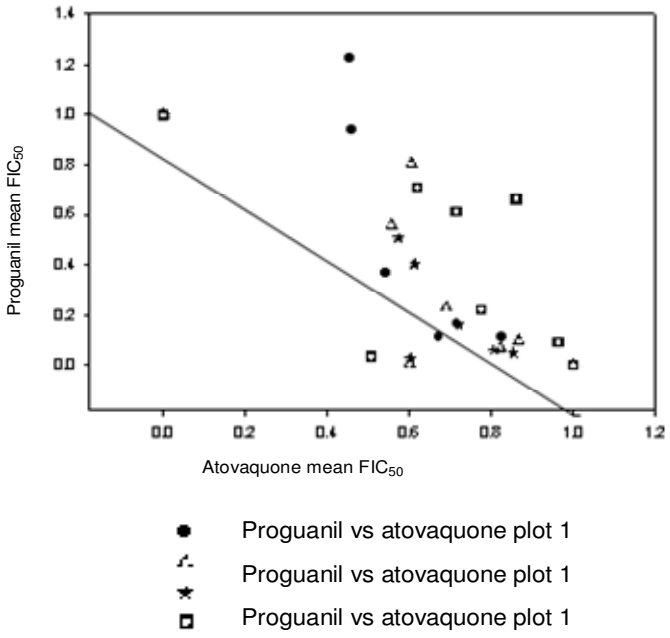


Figure 1. Scatter plot for proguanil/atoquone combination against D6 strain *p. falciparum*. Proguanil vs. atoquone plot 1; Proguanil vs. atoquone plot 2; Proguanil vs. atoquone plot 3; Proguanil vs. atoquone plot 4.

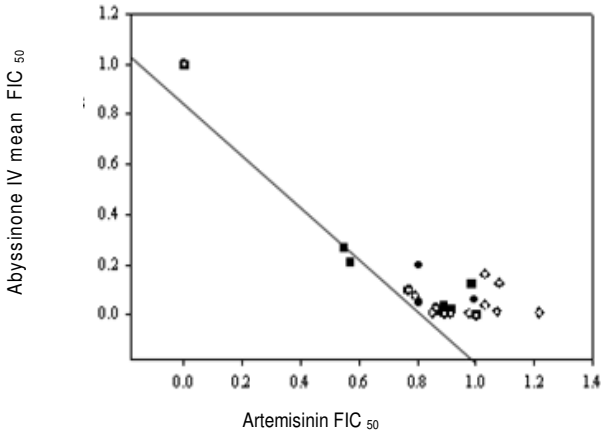


Figure 2. Scatter plot for artemisinin/1 combination.

with artemisinin. The results obtained are summarized in Table 2 and Figures 1 and 3. On the other hand, the results for combination studies of flavonoids 1 - 8 with CQ are summarized in Tables 3 and 5, and Figures 4 - 8.

Activities were evaluated as mean sum of fifty-percent fractional inhibition concentration (FIC_{50}) and grouped into three categories (Ohrt et al., 2002). Those showing synergism (FIC_{50}<1), those showing additive effect (FIC_{50} =1) and those showing antagonism (FIC_{50}>1). The results obtained illustrate activities ranging from having an additive to antagonistic or synergistic effect in combination, against the chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum.* Both strains of plasmodium had comparable FIC_{50} values irrespective of the flavonoid used.

The control drug combination atovaquone/proguanil (Table 3) displayed a range of responses across the varying combinations ratios (Fivelman et al., 2004; White and Olliaro, 1996). The ratios 1:1, 3:1 and 1:4 showed activity with FIC_{50} values <1 to symbolize synergistic response.

Artemisinin/flavanoids combinations

The activity of the combination of artemisinin and abyssinone IV (1) was diverse, but the two compounds predominantly showed additive activity (Table 3, Figure 2). This was observed at the artemisinin/1 combination ratios of 1:1, 3:1, 4:1 and 5:1 with FIC_{50} values <1. Synergism was most evident at combination ratio 5:1. This ratio caused the highest reduction in amount of individual component required to attain the 100% inhibition of the chloroquine-sensitive (D6) strain of *P. falciparum* (Table 3). The rest of the combination ratios were either additive (1:3) or antagonistic (1:4).

At artemisinin/abyssinone 4′ -methyl ether (3) combination ratios of 3:1, 1:3 and 5:1, the drugs showed additive activities (Table 3) against chloroquine-sensitive (D6) strain of *P. falciparum*. Best activity for this combination was at ratio of 5:1 with the lowest mean FIC_{50} across the set of combinations tested. The other combination ratios had mean FIC_{50} >1 showing antagonism.

Artemisinin is the only antimalarial drug with a notably unique molecular structure (Macreadie et al., 2000). Its mode of action has been described (Giao et al., 2004).

The tenacity of finding new molecules have bred the need to reinforce existing ones through combining them to reduce rate at which parasites develop resistance to these drugs. Indeed, artemisinin has been successfully combined with other antimalarial drugs, and the artemether/lumefantrine combination (Coartem®) is currently a first line antimalarial drug (Bukirwa et al., 2006). Interactions of two phytochemicals, artemisinin and licochalcone A, has been studied against synchronized erythrocytic stages of chloroquine-sensitive 3D7 and chloroquine-resistant RKL 303 strains of *P. falciparum.* These two compounds in combination

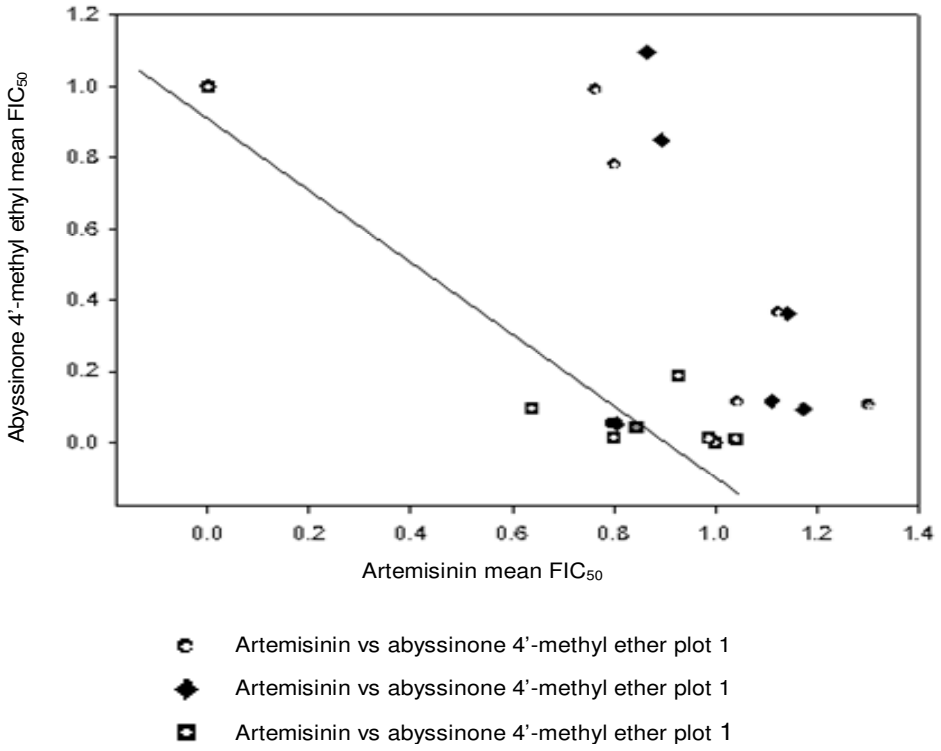


Figure 3. Scatter plots for artemisinin/3 combination activity against W2 strain of *P. falcinarum.*

Table 3. FIC_{50} values for selected artemisinin/flavonoid combinations against D6 strain of *P. falciparum.*

Combining ratio	Mean sum FIC_{50}s ±SD, number of replicates = 3.	
	Artemisinin/1 combination	Artemisinin/3 combination
1:1	0.9 ±0.1	1.1 ± 0.0
3:1	1.0 ± 0.1	1.0 ± 0.1
1:3	1.2 ±0.2	1.0 ± 0.2
4:1	1.0 ± 0.0	1.0 ± 0.2
1:4	1.3 ±0.2	1.0 ± 0.2
5:1	0.6 ± 0.0	1.0 ± 0.1

Number of replicates, n = 3, D6 = chloroquine sensitive strain of *P. falciparum.*

Table 4. Mean sum fractional inhibition concentration (FIC_{50}) for chloroquine/flavonoid combinations activity on D6 strain of *P. falciparum.*

Combining ratio	Mean sum FIC_{50}s ±SD				
	CQ / 2	CQ / 3	CQ / 5	CQ / 7	CQ / 4
1:1	1.6 ± 0.3	1.3 ± 0.4	1.2 ± 0.1	1.2 ± 0.1	3.2 ± 1.8
3:1	1.7 ± 0.2	1.1 ± 0.2	0.6 ± 0.8	1.4 ± 0.0	1.7 ± 0.6
1:3	1.3 ± 0.1	1.4 ± 0.5	1.1 ± 0.8	1.3 ± 0.1	4.6 ± 3.0
4:1	1.4 ± 0.2	1.2 ± 0.2	1.0 ± 0.1	1.4 ± 0.3	1.5 ± 0.3
1:4	1.4 ± 0.1	1.6 ± 0.4	1.2 ± 0.2	1.0 ±0.2	3.6 ± 2.2
5:1	1.3 ± 0.2	1.0 ± 0.1	0.8 ± 0.0	0.7 ± 0.2	0.80 ± 0.1

Number of replicates, n =3, D6 = chloroquine sensitive strain.

Table 5. Mean sum fractional inhibition concentration (FIC_{50}) for chloroquine/flavonoid combinations activity on W2 strain of *P. falciparum*.

Combining ratio	Mean sum FIC_{50}s ±SD					
	CQ / 2	CQ / 3	CQ /5	CQ/6	CQ / 7	CQ / 8
1:1	1.8 ± 0.1	1.3 ± 0.1	1.8 ± 0.5	1.3 ± 0.4	2.4 ± 0.3	2.6 ± 0.3
3:1	1.9 ± 0.1	1.2 ± 0.1	1.7 ± 0.4	1.3 ± 0.2	1.9 ± 0.6	2.1 ± 0.1
1:3	1.7 ± 0.4	1.4 ± 0.1	2.1 ± 0.7	1.4 ± 0.5	1.7 ± 0.1	1.6 ± 0.6
4:1	1.8 ± 0.1	1.1 ± 0.1	1.7 ± 0.1	1.2 ± 0.2	1.7 ± 0.4	1.4 ± 0.1
1:4	1.2 ± 0.0	1.3 ± 0.1	2.1 ± 0.7	1.6 ± 0.4	1.3 ± 0.4	1.2 ± 0.1
5:1	1.4 ± 0.5	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.4 ± 0.2	2.2 ± 0.8

Number of replicates, n=3, W= chloroquine resistant strain.

showed synergistic antiplasmodial activity *in vitro* on these strains (Mishra et al., 2008). However, the present results show potent antagonistic activity for the tested artemesinin/flavonoid combinations against *P. falciparum,* with synergistic and additive effects only being observed at higher artemisinin ratios.

Emergence of drug-resistant *P. fal*ciparum strains to conventional first-line antimalarial drugs has compelled many countries to reorient their drug policies to adopt artemisinin-based combination therapies (ACTs) for treatment of uncomplicated malaria. This has increased the demand of artemisinin, already a scarce commodity. Extensive use of available ACTs will invariably lead to emergence of resistance to these combinations. Thus, there is need to search for new artemisinin-based, inexpensive, synergistic combinations to reduce dependence on artemisinin. The current study indicates that the tested flavonoids do not act synergistically with artemisinin at low ratios. Notably these favonoids have either category A or B activities antiplasmodial activities.

Chloroquine/flavonoid combinations

The results of CQ/ flavonoid combination studies are presented in Tables 4 - 5, and some of the isobolagrams in Figures 4 - 7. Compound 5 in combination with chloroquine yielded additive to synergistic response (Figure 7), while the other flavonoids with chloroquine combinations were mainly antagonistic against the strains of *P. falciparum*. Synergistic response was seen at chloroquine/ 5 ratios of 5:1 and 3:1 with the latter being the best overall chloroquine/flavonoid combination activity observed in this study (Table 4), against CQ D6. Interestingly, 2 singly showed the best activity, among all the flavonoids tested in this study (Table 1), against both strains of *P. falciparum* yet 2 exhibited antagonistic interactions with CQ. This raises questions on the interaction and mechanism of action of these compounds. Chloroquine mechanism of action is mainly through oxidative stress (Domarle et al., 1998) while flavonoids are antioxidants (Liu et al., 2003). Notably, abyssinone 4′-methyl ether (3) had category B activity against both *P. falciparum* strains; yet again it showed antagonistic interactions. The interaction of chloroquine with shinpterocarpan (8) against chloroquine-resistant (W2) strains of *P. falciparum* was indifferent but tended more towards antagonism (mean sum FIC_{50}s >1). Ironically, compound 8 singly, showed category A but did not augment the activity of chloroquine. Such antagonistic responses have been shown in mefloquine/artemisinin combinations, which are singly very effective against *P. falciparum* but antagonistic in combination (Gupta et al., 2002).

Studies on the binding affinity of chalcones to ferriprotoporphyrin IX (FP) versus antiplasmodial activity have revealed a negative correlation (Elena, 2003). Although the activity patterns portrayed by chalcones in this study against the various strains of *P. falciparum* were similar to that of reference drugs that act by impairing binding of (FP) to haemozoin, it is likely that antiplasmodial activities of chalcones may be mediated by other means.

Activity of selected flavonoid against *P. falciparum* MO15-related protein kinase (pfmrk)

In addition to antiplasmodial activity, selected flavonoids were screened against cyclin dependent Kinase (CDK) pfmrk. Protein kinases are a family of enzymes whose key function is involvement in signal transduction for all organisms. This makes it a very attractive target for therapeutic interventions. This enzyme has been targeted in many diseases such as cancer, diabetes, inflammation, arthritis (Vieth et al., 2004) and lately protozoan parasites, specifically *P. falciparum* (Doerig, 2004). Recently several highly specific cdk inhibitors have been described. Olomoucine has been tested on a wide range of protein kinases, and shown to act very specifically on most members of the CDK family with IC_{50} values in the range of 3 - 50 uM (Vesely et al., 1994). Also, 0.2 µM flavopiridol arrests the cell cycle of breast carcinoma cells at the G/M transition and inhibits purified sea star cdc2

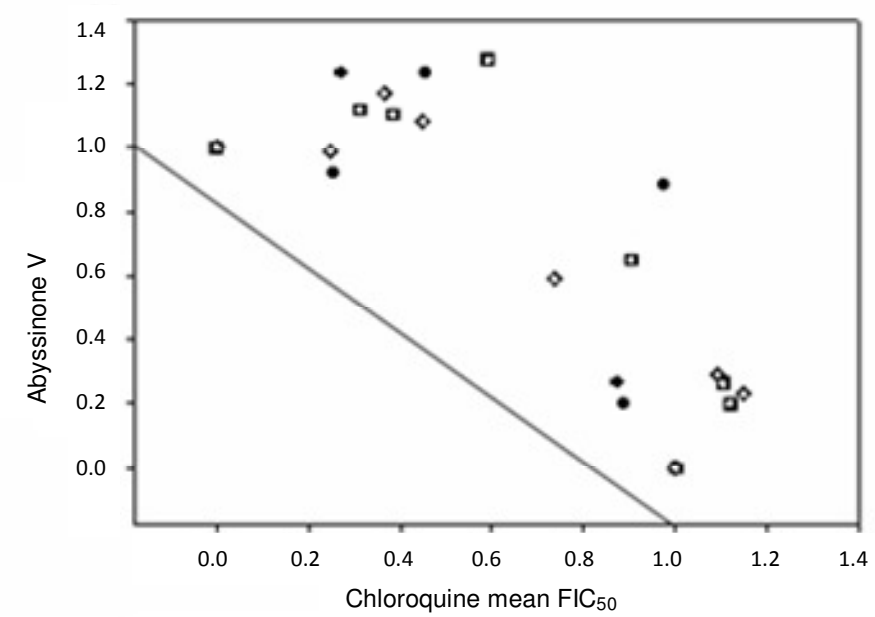


Figure 4. Scatter plot for chloroqine/2 combination against D6 strain *P. falciparum.*

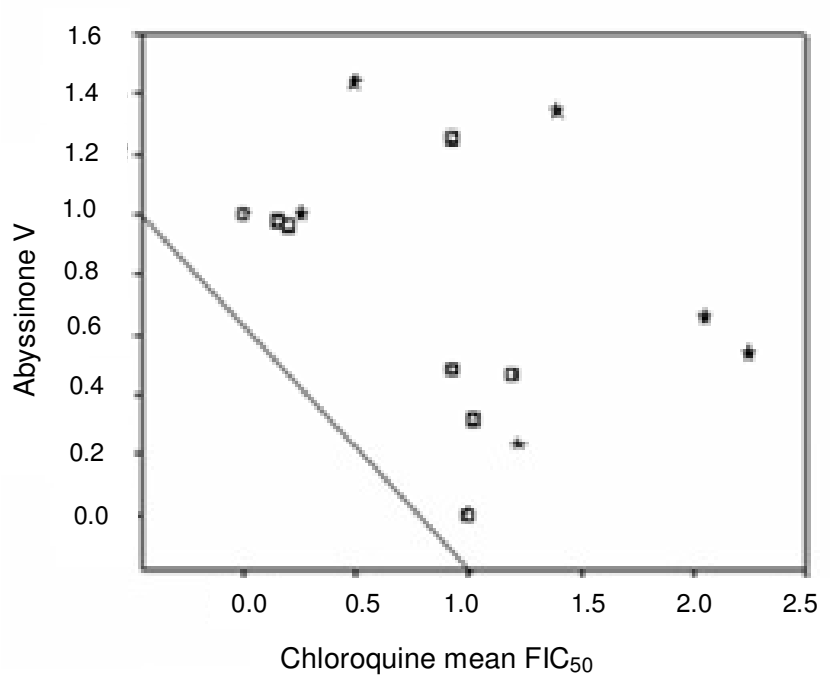


Figure 5. Scatter plot for chloroquine/2 combination activity against W2 strain of *P. falciparum.*

kinase with an IC_{50} of 0.5 µM (Worland et al., 1993). Both drugs: Olomoucine and flavopiridol, act as CDK-specific ATP-analogues (Vesely et al., 1994). These two compounds have shown inhibition of kinase activity of PfPK5

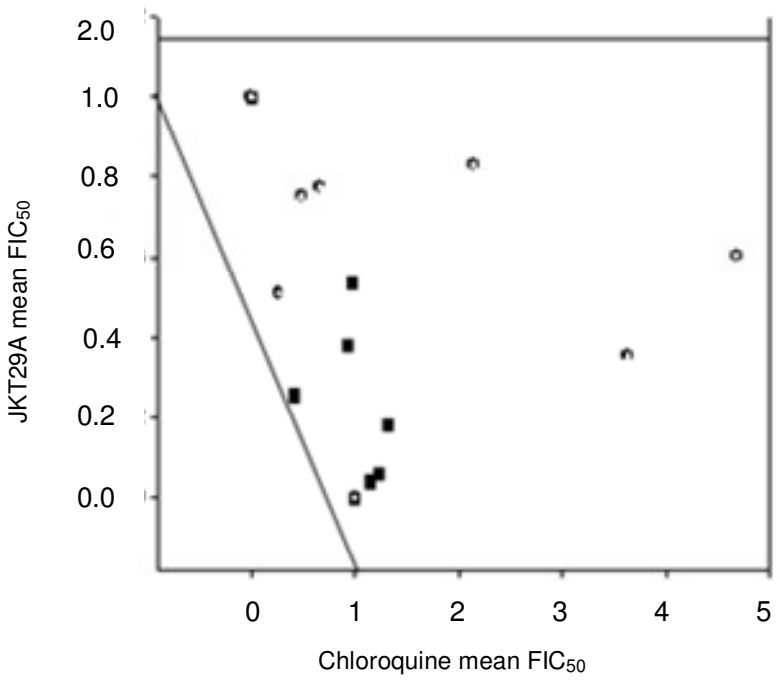


Figure 6. Scatter plot for chloroquine/6 combination against D6 strain *P. falciparum*. JKT 29A = Abyssinone-4'-methyl ether diacetate.

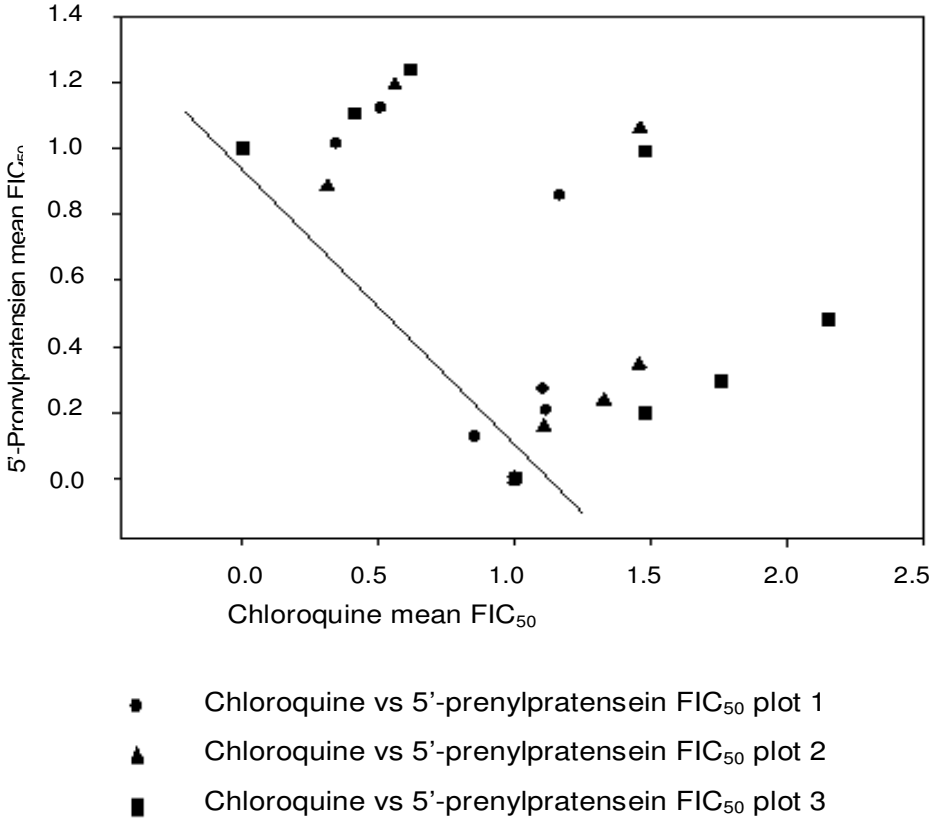


Figure 7. Scatter plot for chloroquine / 5 combinations against W2 strain of *P. falciparum*

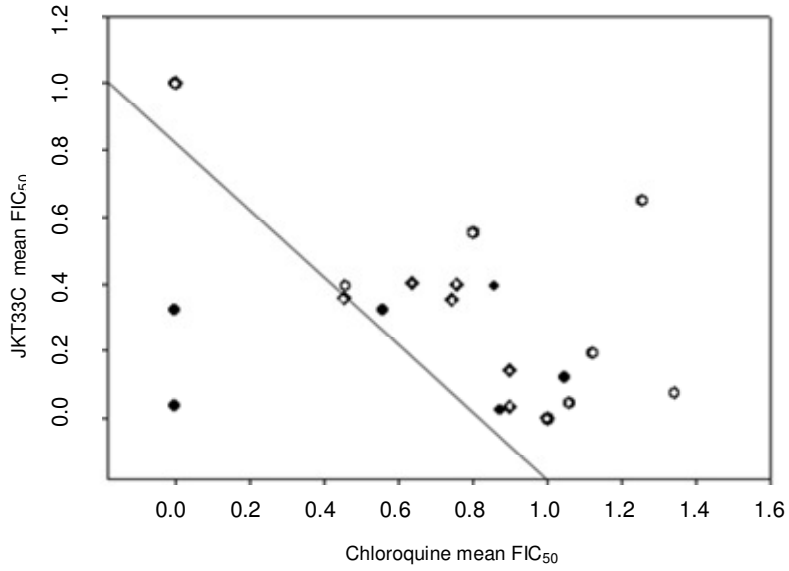


Figure 8. Scatter plot for chloroquine/5 combination against D6 strain *P. falciparum*. JKT 33C = 5 = Sigmoid E.

Table 6. *In vitro* IC_{50} of flavonoid activity against CDK-pfmrk.

Test compound	Anti-pfmrk IC_{50}
	µM
Abyssinone IV (1)	0.05
Abyssinone V (2)	0.04
Abyssinone V-4′ - methylether (3)	>0.24

(IC_{50} values flavopiridol 0.06 µM, olomoucine 0.000015 µM [15 PM]), as well as inhibition of growth of parasite cultures (IC_{50} values flavopiridol 2 µM, olomoucine 0.000015 µM [15 PM]) (Graeser et al., 1996). In this study, compounds that have very good antiplasmodial activities of category A (26, 27, 69), were screened against CDK Pfmrk.

Interestingly in this study, many compounds that have good *in vitro* activity against the *P. falciparum* parasites are effective inhibitors of the *pf*mrk. Notably, abyssinone V (27) that had the best antiplasmodial activity also showed the best inhibition potency. It is interesting to note that when the 4′ -OH in abyssinone IV (26) is methylated (abyssinone V-4′-methyl ether 28) the pfmrk IC_{50} increased more than five-fold showing the importance of free -OH at C-4′.

Compounds 1 - 3, with category A antiplasmodial activity, were tested for their pfmrk activities. The results of the pfmrk assays are listed in Table 6.

Flavonoids 1 and 2 have good *in vitro* activity against the *P. falciparum* parasites and were found to be effective inhibitors of the pfmrk (Table 5). Notably, abyssinone V (2) that had the best antiplasmodial activity also showed the best inhibition (IC_{50} of 0.038 µM), while chalcone (3), did not show anti-pfmrk activity within the range of concentration (highest ≈ 0.3 µM). It is important to note that when the 4′ -OH in abyssinone IV (1) is methylated (abyssinone V-4′-methyl ether 3) the pfmrk IC_{50} increases more than five-fold and hence indicates the importance of free -OH at C-4′.

Conclusion

The flavonoids tested in this study probably have a mechanism of action similar to chloroquine since they have better activity against the chloroquine-sensitive (D6) than the chloroquine-resistant (W2) strain of *P. falciparum*. In addition, the most active flavonoid (2) in combination with chloroquine was antagonistic across most ratios. In addition, the most active flavonoid in combination with chloroquine showed antagonistic activity across all ratios. Since flavonoids are antioxidants, it is possible that as strong oxidants, they may scavenge the chloroquine that is needed to inhibit heme binding thus, leading to the observed antagonism. The chloroquine/ flavonoid and artemisinin/flavonoid combination ratio of 5:1 was the best combination giving synergistic response in most of the tested combinations. It is therefore possible that antiplasmodial activity of flavornoids could also be mediated by pfmrk inhibition since the molecules with the best antiplasmodial activity proved to be effective pfmrk inhibitors.

ACKNOWLEDGEMENTS

The authors are grateful to Institute of Tropical Medicine and Infectious Diseases (ITROMID-JKUAT), University of Nairobi (Chemistry department), The authors are also grateful to Bondo University College and South Eastern University College for their support and lastly the Walter Reed Project /Kenya Medical Research Institute (KEMRI) and Kigali Institute of Science and Technology (KIST) for providing the funds for the study.

REFERENCES

Bell A (2005). Antimalarial drugs synergism and antagonism: Mechanistic and clinical significance. Microb. Lett., 253: 171-184.

Brandao MG, Krettli AU, Soares LS, Nery CG, Marinuzzi HC (1997). Antimalarial activity of extracts and fractions from *Bidens pilosa* and other *Bidens species* (Asteraceae) correlated with the presence of acetylene and flavonoid compounds J. Ethnopharmacol., 57: 131-138.

Bukirwa H, Yeka A, Kamya MR, Talisuna A, Banek K, Bakyaita N, Rwakimari JB, Rosentha PJ, Wabwire-Mangen F, Dorsey G, Staedke S (2006). Artemisinin Combination Therapies for Treatment of Uncomplicated Malaria in Uganda. PLoS Clin. Trials. *1*.

Canduri F, Perez PC, Caceres RA, Filgueira de Azevedo W (2005). CDK9 a potential target for drug development. Comb Chem. High Throughput Screen, 8: 27-38.

Chulay JD, Haynes JD, Diggs CL (1983). *Plasmodium falciparum*: Assessment of *in vitro* Growth by [3H] hypoxanthine Incorporation. Expt. Parasit., 55: 138-146.

Clark AM (1996). Natural products as a resource for new drugs. Pharm. Res., 13: 8.

Desjardins ER, Canfield CJ, Haynes JD, Chulay JD (1979). Quantitative Assessment of Antimalarial Activity *In Vitro* by a Semiautomated Microdilution Technique, Antimicrob. Agents Chemother., 16: 710-718.

Doerig C (2004). Protein kinases as targets for anti-parasitic chemotherapy Biochim. Biophy. Acta, 1697: 155-168.

Domarle O, Blampain G, Agnaniet H, Nzadiyabi T, Lebibi J, Brocard J, (1998). *In Vitro* Antimalarial Activity of a New Organometallic Analog, Ferrocene-Chloroquine. Antimicrob. Agents Chemother., 42: 540-544.

Elena LMS (2003). Interaction of Chalcones with Ferriprotoporphyrin IX Department of Pharmacy, National University of Singapore. http://www.ntu.edu.sg/eee/urop/congress2003/Proceedings/abstract/

Fivelman QL, Adagu IS, Warhurst DC (2004). Modified fixed-ratio isobologram method for studying *in vitro* interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of *Plasmodium falciparum*. Antimicrob. Agents Chemother., 48: 4097-4102.

Francois G, Ake AL, Holenz, J, Bringmann G (1996). Constituents of *Picralima nitida* display pronounced inhibitory activities against asexual erythrocytic forms of *Plasmodium falciparum in vitro*. J. Ethnopharmacol., 54: 113-117.

Freiburghaus F, Kaminsky R, Nkunya MHH, Brun R (1996). Evaluation of African medicinal plants for their *in vitro* trypanocidal activity; J Ethnopharmacol., 55: 1-11.

Geyer JA, Keenan SM, Woodard CL, Thompson PL, Gerena L, Nichols DA, GutteridgeEG, Waters NC (2009). Selective inhibition of Pfmrk, a *Plasmodium falciparum* CDK, by antimalarial 1,3-diaryl-2-propenones Bioorgan. Med. Chem. Lett., 19: 1982-1985.

Giao PT, de Vries PJ, Hung LQ, Binh TQ, Nam NV, Kager PA (2004). CV8, a new combination of dihydroartemisinin, piperaquine, trimethoprim and primaquine, compared with atovaquone–proguanil against *falciparum* malaria in Vietnam. Trop. Med. Inte. Health, 9: 209-216.

Gupta S, Thapar MM, Wernsdorfer WH, Björkman A (2002). *In vitro* interactions of artemisinin with atovaquone, quinine and mefloquine against *Plasmodium falciparum*. Antimicrob. Agents Chemother., 1510-1515.

Keenan SM, Geyer JA, Welsh WJ, Prigge ST, Waters NC (2006). Rational inhibitor design and iterative screening in the identification of selective plasmodial cyclin dependent kinase inhibitors. Biochimica Biophysica acta, 1754: 160-170.

Kokwaro JO (1993). Medicinal plants of East Africa, second Edition; Kenya Literature bureau, p. 56.

Krishna S, Woodrow CJ, Staines HM, Haynes RK, Mercereau-Puijalon O (2006). Re-evaluation of how artemisinins work in light of emerging evidence of in vitro resistance. Trends Mol. Med., 12(5): 200-2005.

Li JL, Robson KJ, Chen JL, Targett GA, Baker DA (1996). Pfmrk, a MO15-related protein kinase from Plasmodium falciparum. Gene cloning, sequence, stage-specific expression and chromosome localization. Eur. J. Biochem., 241: 805-813.

Liu M, Wilairat P, Croft SL, Tan AL, Go M (2003). Structure–Activity Relationships of Antileishmanial and Antimalarial Chalcones. Bio. Med. Chem., 11: 2729-2738.

Macreadie I, Ginsburg H, Sirawaraporn W, Tilley L (2000). Antimalarial Drug Development and New Targets, Parasit. Today, 16: 10-15.

Mishra LC, Bhattacharya A, Bhasin VK (2008). Phytochemical licochalcone A enhances antimalarial activity of artemisinin *in vitro*. Acta tropica, 109(3): 194-198.

Muregi FW, Chhabra SC, Njagi EN, Lang'at-Thoruwa CC, Njue WM, Orago AS, Omar SA, Ndiege IO (2004). Anti-plasmodial activity of some Kenyan medicinal plant extracts singly and in combination with chloroquine. Phytother. Res., 18: 379-384.

Mutabingwa T, Nzila A, Mberu E, Nduati E, Winstanley P, Hills E, Watkins W (2001). Chlorproguanil-dapsone for treatment of drug-resistant *falciparum* malaria in Tanzania. Lancet, 358: 1218-1223.

Nicholas JW (2004). Antimalarial drug resistance. J. Clin. Invest., 113: 1084-1092.

Nzila AM, Nduati E, Mberu EK, Hopkins SC, Monks SA, Winstanley PA Watkins WM (2000). Molecular evidence of greater selective pressure for drug resistance exerted by the long-acting antifolate Pyrimethamine/Sulfadoxine compared with the shorter-acting chlorproguanil/dapsone on Kenyan *Plasmodium falciparum*. J. Infect. Dis., 181: 2023-2028.

Ohrt C, Willingmyre GD, Lee P, Knirsch C, Milhous W (2002). Assessment of Azithromycin in Combination with other Antimalarial Drugs Against *plasmodium falciparum In Vitro*, Antim. Agents Chemother., 46: 2518-2524.

Robert GR (2003). Twenty-seven years of WHO/TDR experience with public–private partnerships in Product R&D for neglected diseases. EMBO reports, 4: 43-46.

Roch KL, Sestier C, Dorin D, Waters N, Kappesi B, Chakrabarti D, Meijer L, Doerig C (2000). Activation of a *Plasmodium falciparum* cdc2-related Kinase by Heterologous p25 and Cyclin H. J. Biol. Chem., 275: 8952-8958.

Taylor WR, White NJ (2004). Antimalarial drug toxicity: a review; Drug Saf., 27(1): 25-61.

Trager W, Jansen BJ (1997). Continuous culture of plasmodium falciparum: Its impact on malaria research. Int. J. Parasit., 29: 989-1006.

Vieth M, Higgs RE, Robertson DH, Shapiro M, Gragg EA, Hemmerle H (2004). Kinomics-structural biology and chemogenomics of kinase inhibitors and targets. Biochim. Biophys. Acta, 11: 243-257.

White N, Olliaro P (1996). Strategies for the prevention of antimalarial drug resistance: rationale for combination chemotherapy for malaria. Parasitol. Today, 12: 399-401.

Yenesew A, Derese S, Irungu B, Midiwo JO, Waters NC, Liyala P, Akala H, Heydenreich M, Peter MG (2003a). Flavonoids and isoflavonoids with anti-plasmodial activities from the roots of *Erythrina abyssinica*. Planta Medica, 69: 658-661.

Yenesew A, Derese S, Midiwo JO, Bii CC, Heydenreich M, Peter MG (2005). Antimicrobial flavonoids from the stem bark of *Erythrina burttii*. Fitoterapia, 76(5): 469-472

Yenesew A, Derese S, Midiwo OJ, Oketch-Rabah HA, Lisgarten J, Palmer R, Heydenreich M, Peter MG, Akala H, Wangui J, Liyala P Waters NC (2003c). Anti-Plasmodial Activities and X-Ray Crystal Structures of Rotenoids from *Millettia Usaramensis* Subspecies *Usaramensis*. Phytochem., 64: 773-779

Yenesew A, Induli M, Derese S, Midiwo JO, Heydenreich M, Peter M G, Akala H, Wangui J, Liyala P, Waters NC (2004). Anti-plasmodial flavonoids from the stem bark of *Erythrina abyssinica*. Phytochemistry, 65: 3029-3032.

Yenesew A, Irungu B, Derese S, Midiwo JO, Heydenreich M, Peter MG (2003b). Two prenylated flavonoids from the stem bark of *Erythrina burttii*. Phytochemistry, 63: 445-448.

Ziegler HL, Hansen HS, Staerk D, Christensen SB, Ha¨gerstrand H, Jaroszewski1 JW (2004). The Antiparasitic Compound Licochalcone A is a Potent Echinocytogenic Agent that Modifies the Erythrocyte Membrane in the Concentration Range where Antiplasmodial activity is observed. Antim. Agents Chemother., 48: 4067-4071.