

Antiplasmodial and chloroquine resistance reversal effects of chalcon derivatives against *Plasmodium falciparum* K1

Zaid OI¹, Abd Majid R², Basir R³

Abstract—Selection of chloroquine (CQ) resistance among different strains of *Plasmodium falciparum* is the worst incident that has ever faced the dedicated efforts to eradicate malaria as CQ is still the safest, most efficacious and cost effective among other anti-malarials. This behooved the scientists to search for other alternatives or sensitizers that augment its action. In this experiment, the potential of six chalcon derivatives; namely; chalcon, 2',4'-dihydroxychalcon, 2',4'-dimethoxychalcon, 4'-chlorochalcon, 4-isopropyl-4'-methylchalcon and 4'-methylthiochalcon to inhibit the growth and reverse CQ resistance of *Plasmodium falciparum* was screened using SYBRE green-I based drug sensitivity assay and isobologram technique. Results show that all the mentioned chalcons have a limited action against *Plasmodium falciparum* K1. Some of the combinations of chalcons with CQ showed an additive action while synergistic effect was seen only for 4'-methylthiochalcon. These actions were not related to their effect on RBCs membrane or to the parasite induced new permeation pathway. Further studies are required to elucidate their full mechanism of actions and if they can augment CQ action in vivo.

Keywords—Isobologram, chalcon, chloroquine, resistance, *falciparum* and SYBR green-I.

I. INTRODUCTION

IN spite of the achieved progress in parasitic ailments eradication, malaria is still a major therapeutic challenge and a significant economic burden in the developing countries due to emergence of drug resistance among different strains of *Plasmodium falciparum*. Furthermore, the debilitating adverse effects of most anti-malarials, limited success to formulate a potential vaccine and paucity of effective alternatives have augmented the dilemma as well [1] and [2]

CQ is still the most pertinent anti-malarial due to its relative safety and efficiency in comparison to other conventional anti-malarials. Furthermore, its cheap price had encouraged the health authorities to rely on it especially in the malaria disseminated poor third world developing countries [3]

Unfortunately CQ started to lose its token due to emergence of both CQ resistance and tolerance among

different strains of *Plasmodium falciparum* [4] and [5]. The former reduces the response to higher doses of the drug while the latter increases the prevalence of the disease re-occurrence [4] and [5]. This issue has urged the scientist to search for other alternatives or chemosensitizers that reduce its resistance [6].

In our experiment, both the anti-plasmodium and CQR reversal potencies of the mentioned chalcons were tested against *Plasmodium falciparum* K1.

Over the recent past, many modern medicines have been inspired and derived from natural products. For instance, in 1970s, the malariologists started to implement artemisinin; a natural compound obtained from the shrub *Artemisia annua*. Other natural products or their synthetic derivatives were screened on continuous *In Vitro* cultures of *Plasmodium falciparum* and were proposed to be introduced in malaria therapy as the main treatment or partner drugs.

Chalcons are highly abundant naturally occurring diaryl ketones (figure 1). They are synthesized easily using one of the established methods, viz; Claisen Schmidt condensation, ultrasonic radiation based or microwave based methods [7]. Moreover, they are set as interesting pharmacophores with plenty of pharmacological activities ranging from a chemotherapeutic action against bacteria, fungi, viruses [8] and cancer cells to an anti-inflammatory and organ protective actions which are mainly attributed to their antioxidant power. This behooved the scientists to synthesize a variety of chalcon derivatives and evaluate their therapeutic effects. Previous studies had pointed out to the discrepancy in the anti-plasmodium effect of different chalcon structures and have highlighted the significance of acetophenone ring substitution (ring B) (figure 1) with some group, viz; methoxy, alkoxy, prenyl or azole.

In our experiment, six chalcon derivatives, namely; chalcon, 2', 4'-dihydroxychalcon, 2',4'-dimethoxychalcon, 4'-chlorochalcon, 4-isopropyl-4'-methylchalcon and 4'-methylthiochalcon were used to investigate their anti-plasmodium and CQ resistance reversing actions against *Plasmodium falciparum* K1. Isobologram technique was used to determine the plausible synergistic, antagonistic or additive interactions of the combination of each with CQ [9]. In order to get some clues about their mode of action, their impact on sorbitol induced hemolysis of the parasitized cell (PRBCs) was screened. Furthermore, their antioxidant potential was tested *in vitro* and compared with its anti-plasmodium effect. In

Zaid OI¹, is with school of medicine and health science, Universiti Putra Malaysia, Selangor 47300

Abd Majid R², is with school of medicine and health science, Universiti Putra Malaysia, Selangor 47300

Basir R³ is with school of medicine and health science, Universiti Putra Malaysia, Selangor 47300

addition, their cytotoxicity against RBCs and mammalian cells were tested to exclude any potential toxicity against normal cells.

II. METHODOLOGY

Materials and chemicals

Human O+ blood was donated by the first author. RPMI-1640 medium, albumax II, were procured from Gibco BRL (Grand Island, NY, USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), triton X-100, sorbitol, hypoxanthine, (100X) phosphate buffered saline (PBS), chloroquine diphosphate (CQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gentamicin was purchased from Jiangxi Dongxu Chemical Technology Co., Ltd.

Six chalcon derivatives were used in this study, namely; 2',4'-dimethoxychalcon, 2',4'-dihydroxychalcon, 4'-chlorochalcon, 4-isopropyl-4'-methylchalcon, 4-methyl-4'-methylthiochalconas well as chalcon; the basic un-substituted form of the compound family. All chalcones were chosen so that they have different substitution on ring B based on what had been previously reported by previous literature about the significance of such substitution in conferring the anti-plasmodial action of the chalcones. All of them were purchased from Indofine Biochemical Company Inc. (Cat No.; 22-300, CAS1776-30-3, CAS CAS 956-02-5, 021274, CAS19-436 and CAS 94-41-7 respectively).

Materials and chemicals

Human O+ blood was donated by the first author. RPMI-1640 medium, albumax II, were procured from Gibco BRL (Grand Island, NY, USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), triton X-100, sorbitol, hypoxanthine, (100X) phosphate buffered saline (PBS), chloroquine diphosphate (CQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gentamicin was purchased from Jiangxi Dongxu Chemical Technology Co., Ltd.

Six chalcon derivatives were used in this study, namely; 2',4'-dimethoxychalcon, 2',4'-dihydroxychalcon, 4'-chlorochalcon, 4-isopropyl-4'-methylchalcon, 4-methyl-4'-methylthiochalconas well as chalcon; the basic un-substituted form of the compound family. All chalcones were chosen so that they have different substitution on ring B based on what had been previously reported by previous literature about the significance of such substitution in conferring the anti-plasmodial action of the chalcones. All of them were purchased from Indofine Biochemical Company Inc. (Cat No.; 22-300, CAS1776-30-3, CAS CAS 956-02-5, 021274, CAS19-436 and CAS 94-41-7 respectively).

Blood washing

Once it was donated, the blood was centrifuged at 3000 RPM for 5 min and washed thrice with incomplete RPMI-1640 (RPMI-1640, 20µg/ml gentamicin and 25 mM HEPES); kept in the fridge overnight and used for not more than one week after its preparation [10].

Parasite culturing, maintenance and synchronization

Plasmodium falciparum K1(ATCC), procured from the Institute of Medical Research, Kuala Lumpur Malaysia, was cultured in O+ red blood cells suspended in a Complete Malaria Culture Medium (cMCM) containing RPMI-1640, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer (pH 7.4), 0.75 mM hypoxanthine, 0.5% albumax, 24 mM sodium bicarbonate, 11 mM glucose and 50 µg/L gentamicin. Both pH and hemotocrite were maintained at 7.4 and 2% respectively. The culture was incubated at 37 °C in a micro-aerophilic atmosphere containing 90% N₂, 5% CO₂ and 5% O₂. The medium was changed every 24 hrs and the growth was checked using Giemsa stained thin blood smears [11] and [10].

Parasite synchronization

Parasite synchronization was performed as previously described (Venderberge et al 1979). Briefly, the pelleted unsynchronized parasitized red blood cells (PRBCs) were incubated with an equal volume of 5% (w/v) sorbitol solution for 10 minutes. Sorbitol was washed out thrice using RPMI-1640 washing medium (RPMI-1640, 25 mM HEPES and 50 µg/ml gentamicin). Sorbitol lyses all the PRBCs that contain the parasite at the trophozoite or schizont stages leaving those which are at their ring stage. It is preferred to establish this step during preponderance of the ring stage. Finally, the washed PRBCs are cultured in cMCM for further analysis [12].

Stock solution preparation

Stock solutions of 10 mM of each chalcon and CQ were prepared using methanol for the former and PBS (pH 7.4) for the latter.

Malaria drug sensitivity assay

Malaria drug sensitivity assay was performed as previously described by Mathias et al 2010. Drug containing flat bottomed 96 well microtiter plates; featured the triplicate of two folds serial dilution of CQ (1 nM- 1 µM) or chalcones (1 nM to 250 µM), were incubated for 48hrs at 37 °C with PRBCs (Parasitized Red Blood Cells). Control wells, containing drug without RBCs, untreated RBCs (0% parasite growth) and untreated PRBCs (100% parasite growth) were allocated as well. The parasites were synchronized at the ring stage and maintained at a final hematocrite (Hct) and parasitemia of 1%. The final volume was 100µL (50 µl of drug dilution+ 50 µl of PRBCs suspension). Working solutions were prepared using cMCM at a final concentration equal to the highest dilution. The test was done in triplicate. After incubation, the plates were freeze-thawed and 100 µL of SYBR green-I lysis buffer ((20 mM Tris, 5 mM EDTA, 0.008% saponin and 0.008% triton-X-100) was loaded to each well. Then the plates were incubated at room temperature for 1 hr. and finally fluorescence was measured twice after 15 seconds of plate agitation using Victor Plate reader (Perkin Elmer, Salem, MA) at an excitation/ emission wavelength of 485/535 nm. Geometric mean of the first and second pass was used to exclude any measurement error [13].

We were restricted to 250 nM as it was the maximum concentration at which some of our lipophilic chalcones; viz, chalcon, were miscible with cMCM. Although, the hydroxylated and alkoxyated ones were miscible at higher concentrations.

Estimation of IC₅₀ and IC₉₀

Both IC₅₀ and IC₉₀ of each test drug against *Plasmodium falciparum* K1 were determined using Microsoft excel 2007 software according to the recommended protocol of percentage of parasite inhibition versus log [drug concentration].

Drug combination assay and isobologram analysis

For drug combination assay, working solutions of each test compound were prepared from their stocks at concentrations equivalent to 16 times their IC₅₀. The dilution was chosen such that IC₅₀ of each falls in the fourth twofold serial dilution. Then the two solutions were mixed once at fixed ratios (10:0, 7:3, 5:5, 3:7 and 0:10; ratios of CQ/phytochemical in nM respectively). Then each combination was uploaded in triplicate in row H of the 96 well plates (cells 2-11) and were serially diluted throughout the plate (row G-B). The peripheral wells were uploaded with drug, RBCs and PRBCs controls (Fig 2) [9]. After that, PRBCs suspension (synchronized at ring stage) was uploaded to the drug combinations containing wells so that the final volume was 100 µL (50 µL of each diluted combination+50 µL of PRBCs suspension). Then the mixture was incubated at the standard conditions for 48 hrs and treated as in drug sensitivity assay to determine parasite growth and assess IC₅₀ and IC₉₀ of each combination separately. For each combination ratio, both FIC₅₀ and FIC₉₀ (fractional inhibitory concentration) were calculated through finding the ratio of IC₅₀ or IC₉₀ of each drug within the combination to that when the drug was incubated with the parasite alone [9].

At the end, two isobologram curves were plotted so the first, FIC₅₀ values for CQ and each chalcon were extrapolated on Y and X axes respectively. Similarly, FIC₉₀ of each were extrapolated for the other curve. The line that links the two drugs FICs is considered as line of additivity such that, the interaction is considered additive when the points fall on the line, synergy and antagonism if they fall below or above the line respectively [9].

Effect of the phytochemicals on non-infected RBCs

Different concentrations of each chalcon (1 nM -250µM) were incubated with O +ve human RBCs in an Incomplete Culture Medium iCM (RPMI-1640, 25 mM HEPES and 20 µg/ml gentamicin) at 37 °C for 48 hrs using 24 well plate (1 ml/cell). After incubation, 500 µl from each well was transferred to Eppendorf tubes after through mixing, centrifuged at 500 g for 5 min, 200 µl of the supernatants were loaded into a flat bottomed 96 well plate and the released hemoglobin was measured at 540 nm (VersaMaxtm). Results were compared with both negative and positive controls wherein the RBCs were incubated with drug free media and

a medium containing 1% Tween 20; which produces 100% hemolysis.

Effect of the phytochemicals Vero cells

Vero cells were incubated for 48 hrs with different concentrations of each chalcon (1 nM-300 µM) at 37 °C 5% CO₂, 5% O₂ and 90 % N₂ in a culture medium containing RPMI-1640, 10 % BSA (Bovine serum albumin) and antibiotics ((100 U/ml penicillin and 100 µg/ml streptomycin). After incubation, MTT assay was performed as previously described [14]. Finally dose response curve was extrapolated and IC₅₀ against Vero cells was calculated using Microsoft excel 2007.

Selectivity index

Selectivity index was calculated through finding the ratio of the drug IC₅₀ against plasmodium to that against RBCs and Vero cell line. It is calculated to find the degree of selectivity of the drug toward the parasite as compared to two types of mammalian cells; RBCs, where the plasmodium thrives, and Vero cell, which is chosen to be a representative of human epithelial cells.

Effect of on merozoites invasiveness

Effect on merozoite invasion was performed as previously described [15]. Briefly, non-infected RBCs were treated at 37 °C for 2 hrs with different concentrations of the drug (1 µM-1 mM) dissolved in the incomplete malaria culture medium (RPMI-1640, HEPES-tris buffer 25 mM and gentamicin 20µg/ml). Then the exuberant drug was washed twice and to each 300 µl of the treated RBCs, 100 µl of PRBCs at parasitemia of 4% and rich in the schizont stage (after >35 hrs of parasite synchronization) was added and the final Hct was adjusted at 1%. The mixture was incubated at the mentioned incubation conditions for 20 hrs; the time point at which microscopic determination of parasitemia was done. In this test, the amount of merozoites that could have invaded the treated RBCs was compared to the positive control that contains only non-treated RBCs.

Effect of each chalcon on sorbitol induced hemolysis of PRBCs

Chalcones Effect on parasite induced permeability pathway was investigated as previously described [16]. Briefly, PRBCs predominated with trophozoites (after 30 hrs of synchronization) at 15 % parasitemia were washed and 100 µl of the washed cells were loaded into 24 well plates, featured two folds serial dilution of each chalcon or frusemide; a well known inhibitor of plasmodium induced NPPs, with a concentration range of (500 nM-250 µM) for each at 1 ml /well. The dilutions were done using sorbitol buffer solutions (300 mM sorbitol in 10 mM HEPES-Tris (pH 7.4)). After 30 min incubation at 37 °C, 500 µl was aliquoted from each suspension after through mixing, centrifuged at (500 g for 5 min) and 200 µl of the supernatants were loaded to a flat bottomed 96 well plate. The released hemoglobin was measured at 540 nm (Spectramax plus 384) and the percentage of hemolysis in each sample was compared to the positive control (wells containing the drug free sorbitol

buffer solution) [16]. Finally, IC₅₀ (drug concentration required to inhibit 50% of parasite induced permeability pathway) was obtained by linear regression analysis using Microsoft excel 2007 software. Frusemide, a well known inhibitor of NPP, was used as a negative control at 100 μM [17].

Effect of chalcones on RBCs fragility

RBC fragility test was performed as previously described. Briefly, washed RBCs were suspended in series of solutions containing isotonic PBS diluted serially using D.W so that tonicity ratio would be kept in the range of 0-0.9% noticing that 0% tonicity gives 100 % hemolysis. Then sets of the mentioned dilutions were prepared and each chalcon at two different concentrations (1μM and 250 μM) were served. Finally, percentage of hemolysis vs. tonicity curve was extrapolated for each drug concentration and was compared to that of the drug free set.

Antioxidant activity

The antioxidant activity of the test chalcones was screened using hydrogen peroxide scavenging assay, reducing power assay and DPPH radical-scavenging assay as described by *Ruch et al 1989, Oyaizu, M et al 1986 and Hatano et al 1989* respectively.

Physicochemical properties calculation and bioactivity prediction

Both physicochemical properties and the predicted bioactivity parameters for each chalcon were determined using chemi-informatic software known as Molinspiration (<http://www.molinspiration.com>). It performs a fragment based virtual screening of the following, viz; CLOGP (logarithm of octanol/water partition coefficient), PSA (molecular polar surface area), nON (number of non-hydrogen atoms), nOHNH (number of hydrogen donating bonds) and nrotb (number of rotatable bonds). Furthermore, the software gives predictive drug-likeness score toward the following intracellular targets; GPCR, kinase, nuclear factors, ion channels and protease enzyme.

III. RESULTS

Drug sensitivity assay

Plasmodium falciparum K1 was resistant to CQ (IC₅₀= 265 ± 3.3 μM, IC₉₀= 433 nM). It was poorly responsive to all the mentioned chalcones with IC₅₀ values within the micro molar range as seen in (TABLE 1).

Effect on nRBCs stability

All the used chalcones were cytotoxic to nRBCs as seen in TABLE 2. Only 2',4' dimethoxy-chalcon and 2',4' dihydroxy-chalcon showed good selectivity index SI toward the plasmodium as compared to nRBCs (SI>10). Others, like; chalcon and 4 methoxy-4'thiomethyl chalcon were partially selective with SI value <10 & > 4 while the rest; 4'chlorochalcon and 4-isopropyl-4'methyl chalcon were totally

non selective to the plasmodium as their SI values were < 4 (TABLE 1).

Effect mammalian Vero cells

All of the used chalcones except 2',4' dihydroxychalcon were cytotoxic to Vero cells at a concentration < 1 mM. Each of 4'chlorochalcon, 4'-methyl-4-methylisopropylchalcon and 4-methyl-4'- thiochalcon were more cytotoxic as compared to the others. Three of them showed poor selectivity to plasmodium in comparison to Vero cells; 4' chloro-chalcon, 4'methyl-4-isopropylchalcon and 4-methyl-4'methylthiochalcon. The SI toward plasmodium in comparison to the vero cells was higher than what had been obtained when it was calculated based on the cytotoxic effect on RBCs (TABLE 1)

Due to their higher RBCs hemolytical effect and poor selectivity to the plasmodium, each of isobologram analysis, merozoite invasion assay and the effect on RBCs fragility test were not performed for each of 4'chlorochalcon, 4'-methyl-4-isopropylchalcon and 4-methyl-4'methylthiochalcon

Isobologram analysis

Combinations of CQ with each of the used chalcones did not produce any antagonistic effect. Few combinations produced an additive effect (TABLE 3) while the effect in most of them was indifferent. Only one combination produced a synergistic effect (CQ/4-methyl-4'-methylthiochalcon at 7:3) within the IC₅₀ based isobologram (TABLE 2).

Merozoite invasion inhibition assay

Results of the potential of merozoites to invade the RBCs treated with chalcon, 2',4'- dimethoxychalcon or 2',4' dihydroxy-chalcon are illustrated in figure ---. Effect of the others was not tested due to their detrimental impact on RBCs at low concentrations. Figure 3 illustrates the percentage of parasite duplication inhibition in the cultures containing RBCs treated with test chalcon as compared with that of the cultures containing the untreated RBCs only. Their IC₅₀ as calculated by curve fitting of the percentage of parasitemia versus log (drug concentration) curve was 1.106, 1.846 and 15.021 mM respectively (figures 3A and 3 B).

Effect of chalcones on RBCs osmotic fragility

Effect of each of chalcon, 2',4'-dimethoxychalcon, 2',4'-dihydroxychalcon on RBCs osmotic fragility (O.F) is depicted in figure 4. The figure shows the threshold ionic strength wherein maximum RBCs hemolysis commences was elevated when the mentioned chalcones were used at a concentration of 250 μM. Meanwhile, at 1 μM, no any noticeable action was observed (figure 4).

Physicochemical properties and bioactivity prediction

Results of Molinspiration software (www.molinspiration.com) are illustrated in (TABLE 3) wherein the impact of such substitutions on both the hydrophilicity and the polarity of the molecule. Both ClogP and PSA were obviously increased in the chalcones substituted with lipophilic groups. The upmost increment was

obtained for 4'-methyl-4-isopropyl chalcon. Meanwhile it was less in 4'-methylthiochalcon and 4-chlorochalcon and it was negligible in the methoxylated chalcon. On the other hand, Chalcon hydroxylation at ring B could have reduced the lipophilicity.

The simulation software shows that all the substitutions confer for higher predictive bioactivity against the main intracellular targets; GPCR, kinase, protease and nuclear receptors as represented by drug-likeliness score. The predictive improvement was higher for both the hydroxylated and methoxylated chalcon and less or absent for the halogenated chalcon and its thio-methyl derivative.

Antioxidant activity

All of the tested chalcon did not show any antioxidant activity except for the hydroxylated and methoxyated ones (TABLE 4)

IV. DISCUSSION

Previous studies had pointed out to the role of natural products in eradicating malaria parasite. Few of them were worth to be used in the clinical field, such as; *Artemisia annua*. Research is going on to investigate their plausible anti-plasmodial potential [21]. In our study, we had screened both the anti-plasmodium and the selectivity index toward plasmodium as compared to RBCs and Vero cells for six chalcon derivatives, namely; chalcon, 2',4' dihydroxy-chalcon, 2',4-dimethoxychalcon, 4'-chloro-chalcon, 4-isopropyl-4'-methyl chalcon and 4-methyl-4'-methylthiochalcon against *Plasmodium falciparum* 3D7 and K1. They represent the un-substituted, hydroxylated, methoxylated, halogenated, alkylated chalcon and thio-chalcon derivatives respectively. They were chosen based on the previous literatures that highlighted the significance of ring B substitution of the chalcon nucleus (fig 1) to enhance their anti-plasmodium action [22].

Formerly, it had been suggested that their pharmacological activity is retained within its carbonyl group which entitles the molecule to act as a nucleophile attaching different intracellular targets (figure 1). They target the intracellular enzymes involved in cellular growth or intracellular cellular function regulation [22]. Ubiquity of ring B substituent's alters the physiochemical properties, viz; the polarity or the lipophilicity, of the compound (TABLE 4) resulting in changing the extent of its pharmacological activity or its permeability to the intracellular targets. Furthermore, their potential to undergo the nucleophilic attack on the active sites of the intracellular targets would change as well [22]. On the other hand, they may add an antioxidant power [23]; a character to which the potential of the compounds to mop out the free radicals and combat the oxidative stress is attributed. Antioxidants act as double edged sword weapons for the cells. From one side; they halt flow of the deleterious free radicals; which are released as by products due to the cellular activities. They may turn into pro-oxidants and release more free radicals at higher concentration. This concentration threshold is different between different cells and it is not sure if there is a discrepancy in this threshold between plasmodia and human

cells [24]. Previous studies had pointed out to the significance of such discrepancy in eradicating the undeveloped cells [25] [26] [27].

In our study, all the test chalcon produced an anti-plasmodium effect with IC₅₀s within the micro-molar range which is higher than that of any conventional anti-malarial. Hydroxylation of the B ring at both para and ortho positions did not intensify the anti-plasmodial effect of the chalcon although it augmented its anti-oxidant potential. This excludes any potential for the compound to produce any pro-oxidant effect against *Plasmodium falciparum*. Some previous studies has pointed out to the significance of ring B hydroxylation in potentiating the anti-tuberculosis effect [8] but according to our results, this modification is not advantageous for the anti plasmodial effect of chalcon.

Furthermore, the study shows that the lipophilic chalcon have better tendency to limit the parasite growth as compared to the less lipophilic ones as IC₅₀ values of both the methoxylated and hydroxylated chalcon were lower than that of those substituted with groups of higher lipophilicity. [8] has pointed out to the significance of the lipophilic groups ubiquity on their anti-microbial effect The highest effect was obtained for the thio-chalcon derivative positing a role for sulfur to enhance the anti-plasmodium effect. Nevertheless, none of them has succeeded to inhibit plasmodium growth like any potential anti-plasmodium drug. It is noteworthy that these results came in contrary to what was predicted by Molinspiration chemi-informatic software. The software predicts higher activity for the hydroxylated and methoxylated chalcon and less activity for the thiochalcon derivative. This suggests either presence of other intra-cellular mechanisms through which the latter produces its action or may be its permeation is higher than other chalcon. The results show also that in spite of its higher lipophilicity, 4'-methyl-4-isopropylchalcon has lower antiplasmodial action in comparison to the thiochalcon derivative. This suggests that the former's lipophilicity is higher than the optimum threshold required for the drug penetration into its intracellular target.

All the chalcon derivatives showed good selectivity to plasmodium as compared to mammalian cells. Selectivity to plasmodium was compared to two types of mammalian cells; Vero cells; mammalian kidney epithelial cells obtained from monkeys, and human RBCs. All of them showed cytotoxic effect but not into the level of cytotoxicity that such chalcon produced on plasmodial cells. This points out to the potential safety of these compounds to mammals. Nevertheless, further studies are required regarding this point.

Their cytotoxicity against the mammalian cells can be attributed to their lipophilicity. Their effect on RBCs was higher as compared to Vero cells and it was exclusively higher for those which possess high ClogP and low PSA (TABLE 2 & 4). This suggests that chalcon have the ability to accumulate in the double layered membrane of the RBCs resulting in disruption of their integrity and enhancing their hemolysis. This cytotoxic effect was prominent at concentrations comparable to their anti-plasmodial IC₅₀ for

each of 4'-chlorochalcon, 4'-methyl-4-isopropylchalcon and 4-methyl-4-methylthiochalcon. At such concentrations, all of them produced a pronounced effect on RBCs morphology characterized by echinocytosis and spherocytosis (figure 5). This suggests that the anti-plasmodium effect of these chalcon is attributed mainly to their effect on RBCs rather than their direct effect on plasmodium. This has behooved us to omit them in the other tests.

O.F test was performed only on the chalcon that did not deteriorate the RBCs stability significantly, viz; chalcon, 2',4'-dihydroxychalcon and 2',4'-dimethoxychalcon. All of them have shifted the % of RBCs hemolysis versus ionic strength curve into upward direction indicating higher sensitivity of the chalcon treated RBCs to the hypotonicity induced stress. Furthermore, the shift indicates that the ionic strength threshold; at which RBCs hemolysis commences, goes up the mentioned chalcon. The increment was higher and more significant for the un-substituted and the methoxylated chalcon. This may be attributed to their higher lipophilicity which augments their impact on RBCs membrane function. It is well known that when RBCs encounter the hypotonicity stress, they tend to swell and then hemolyze. Furthermore, it has been also suggested that chalcon may affect cell membrane function through targeting protein channels present on the cell membrane or affecting the membrane stability due to their hydrophobic nature that entitle them to accumulate in the cell membrane [22].

It is noteworthy that some chalcon are used conventionally as model drugs against the plasmodium induced new permeation pathways on PRBCs surface, viz phlorizin. Phlorizin is a famous glycoside with a potential to inhibit glucose entry into the intracellular compartments and its potential to inhibit the plasmodium induced NPPs had been studied extensively. It is not clear whether its action on NPPs and glucose transporters due to its glycosidic nature or its nature as a chalcon derivative [28].

Presence of phenolic substitution on B ring has increased the potential of the chalcon to inhibit the NPPs function as depicted in results of sorbitol induced hemolysis inhibitory assay. Both the hydroxylated and the methoxylated chalcon had their IC₅₀ for the drug effect on NPPs approaches to 10 μM. Presence of the lipophilic groups did not produce any improvement in this regard. This suggests that as chalcon derivatives, they can attack the NPP pathway resulting in inhibition of nutrients influx. Furthermore, their impact on NPP may be due to their tendency to bind to the NPPs rather than accumulation on RBCs membrane and affecting RBCs fluidity [29]. The chemi-informatic data obtained from Molinspiration software suggest that the hydroxylated chalcon have the highest impact on the ion channels as compared to the others (TABLE 3). This observation could not have been obtained during NPP activity assessment and this may be due to the discrepancy in the nature of such channels with that of the NPPs.

Their impact on merozoite invasion was screened through merozoite inhibition assay. All of the tested chalcon; chalcon, 2',4'-dihydroxychalcon and 2',4'-dimethoxychalcon, have reduced the capacity of the

merozoites to invade the treated RBCs in different extent (figure 3). This suggests that chalcon can inhibit merozoite invasion in a manner dependent on their lipophilicity and their ability to accumulate in RBCs membrane. During merozoites invasion, some external merozoite surface antigens, viz; *pfmsp-1* (Plasmodium *falciparum*-merozoite specific antigen) bind to the RBCs Duffy coat resulting in induction of a cascade of sequential processes that ends up with the intracellular entry of the merozoites to the RBCs. The negative impact of such compounds on RBCs integrity or their potential to tolerate stressful situation might have adversely affected the portals through which the parasite invades the RBCs. For the three tested chalcon, this action was obvious at higher levels (figure 3) which are difficult to obtain in plasma due to their poor water solubility.

The interaction of CQ with the three abovementioned chalcon against *Plasmodium falciparum* K1 was studied using the isobologram technique wherein two isobolograms were derived; the IC₅₀ and IC₉₀ based ones. They reflect the plausible potential of each chalcon to reduce both CQ resistance and tolerance respectively. The former is correlated with the inaptitude of the parasite to respond to high doses of the drug while the latter is correlated with period required for parasite eradication [30] and [31] and [5]. Both problems started to encounter different strains of *Plasmodium falciparum* worldwide and predestined for raising the dose and elongating the treatment period. Chemo-sensitizers incorporation into the therapy may facilitate achieving the therapeutic target without subjecting the patient to the haphazard of CQ toxicity. Chemo-sensitizers do not merely affect CQ resistance, tolerance is inhibited as well. For instance, in our results, some of the proposed chemo-sensitizers had succeeded to synergize CQ such that they reduced the IC₉₀ rather than affecting the IC₅₀.

Antagonism was absent in all the combinations as none of them produced a sum for FIC₅₀tot or FIC₉₀> 2 (TABLE 3). Antagonism with CQ may occur in the presence of any agent that interferes with access of CQ to the plasmodial digestive vacuole or inhibits the CQ induced oxidative stress through mopping out the free radicals. These results exclude any possibility for any of the tested chalcon to produce such actions. Furthermore, all the tested chalcon failed to produce any synergy. But the additive effect was seen in some of the combinations and was more prominent in the IC₉₀ based isobologram rather than the IC₅₀ based one. This suggests that addition of such chalcon have better potential to compromise the tolerance of the parasite to CQ rather than affecting its CQ resistance. This imparts the notion that their uptake may help to reduce the period of time required to clear the parasite. For the IC₅₀ based isobologram, the additive effect was seen in both CQ/chalcon and CQ/2',4'-dimethoxychalcon combinations at a ratio of (7:3) but it was absent when the hydroxylated chalcon was combined. On the other hand, the IC₉₀ based isobologram showed higher number of combinations characterized by an additive interaction with a higher extent for the more lipophilic chalcon; chalcon and the methoxylated derivative, as compared to that of the hydroxylated one. Their lipophilicity

may help them to reach their target meanwhile the ubiquity of the electron donating group in the hydroxylated derivative may affect the potential of the carbonyl group to undergo its nucleophilic attack to their target (figure 1). It is noteworthy that all the mentioned substituent groups may reduce the dipole potential of the carbonyl group resulting in less nucleophilic attack to the other enzymes. It is supposed according to Molinspiration information that both the methoxylated and the hydroxylated chalcones have a comparable predictive activity against the mentioned cellular targets and their activity was higher than that of the non-substituted one. This excludes the notion that the claimed action is attributed to their potential to attack the intracellular targets. On the other hand, In spite of their comparable predictive effect in the Molinspiration informatics software, the impact of the former on the plasmodium is more prominent and was comparable to that of the un-substituted chalcon.

Both of the methoxylated and the un-substituted chalcones share one character that they have a comparable CLOGP values indicating that their potential to induce such action emanates from their lipophilic characters rather than their potential to suppress functions of different intracellular targets. Furthermore, according to our results, such difference cannot be attributed to the difference in their impact to inhibit the plasmodial NPPs as all of them had affected them in a comparable extent indicating ubiquity of actions other than their impact on NPPs in potentiating CQ action.

Further studies are recommended to see the interaction of chalcones with CQ in vitro as not merely does emergence of resistant and tolerant phenotypes of *Plasmodium falciparum* hinder s of CQ action. CQ pharmacokinetic characters constitute an obstacle against its success. Previous studies pointed out to the wide fluctuation and inter-individual variations in CQ plasma concentration and other pharmacokinetic parameters when it is administered within the therapeutic dose. These discrepancies result from the tendency of the drug to deposit in lean body mass through binding with melanin and various proteins. This shuffle CQ into sites away from their target and prevent attaining the target therapeutic concentration in plasma [32] and [33].

Overall, chalcones have limited potential to inhibit plasmodial growth and some of them can reduce both CQ resistance and tolerance to a limited extent. In spite of the prominent potential of some of them to inhibit the plasmodium growth, their use in malaria treatment is not applicable as they had deleterious impacts on RBCs stability at concentrations comparable to their IC₅₀s against the plasmodia. It is noteworthy that all of them had a CLOGP values more than 4.2 indicating that the plasmodial selectivity of such chalcones is low and are not recommended to be implemented in malaria therapy. Those with moderate lipophilicity had better selectivity as compared to them and have an additive effect with CQ. Meanwhile, when the lipophilicity of the chalcon derivative was reduced (as in the hydroxylated chalcon), the toxic effect against the plasmodium as well as on RBCs and mammalian cells was reduced.

All of these results suggest that lipophilicity was the major determinant in controlling the behavior of the chosen compounds on plasmodial activity. Moreover, their antioxidant potential does not play a significant role. Furthermore, their effect on RBCs stability or the plasmodium induced new permeation pathway is not highly correlated with their effect on plasmodium.

Further studies are recommended to study the impact of other substitutions on the selectivity of the compound toward the plasmodium as compared to human cell lines as well as their antiplasmodial action. In addition, further in vivo studies are recommended to see if they have also an effect on CQ pharmacokinetic.

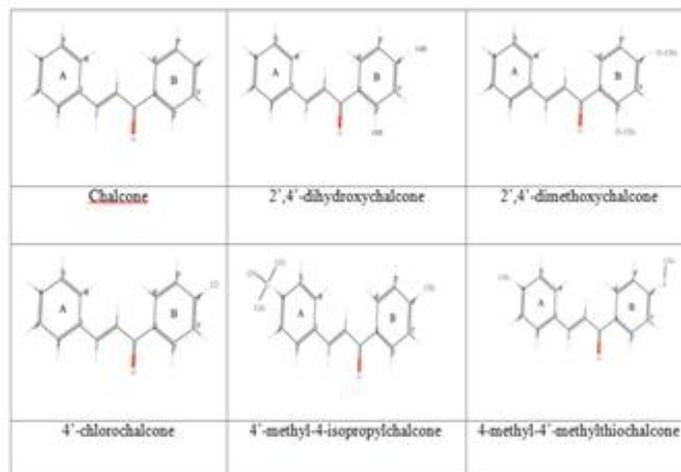


Fig 1 :- Chemical structures of the chalcon and the chalcon derivatives that was used in the study. The structure in the upper left corner represents the structure of the basic chalcon nucleus that is made of a di-aryl keton structure (1,3-Diphenyl-2-propen-1-one). It contains two rings; A & B. Any substitution on ring B is tagged by "R". Substitutions on ring A affect the electron density on the carbonyl group resulting in changing their ability to attack nucleophilic groups. The other structures represent the substituted chalcones that has been used in the study.

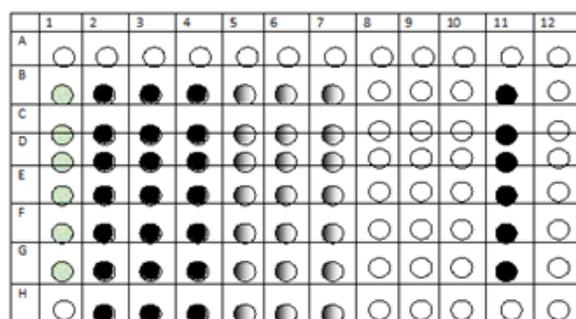


Fig 2:- Isobologram layout on flat bottomed 96 well plate with concentration ratios of CQ to each of the test phytochemical as three solutions (with CQ/phytochemical ratios 7:3, 5:5 and 3:7 respectively). When the plates were prepared as described in the text, wells tagged with ○ serve as an RBC control (no drug and no parasites; 100% growth inhibition), black wells serve as a parasite control (no drug; 0% growth inhibition), wells tagged with ○ and ○ serve as the serially diluted CQ/test phytochemical mixtures at ratios 7:3, 5:5 and 3:7 respectively with the wells in row H holding the highest and served as drug control

(No RBCs at all). Another 96 well plate was prepared similarly containing the serial dilution of each drug solution separately

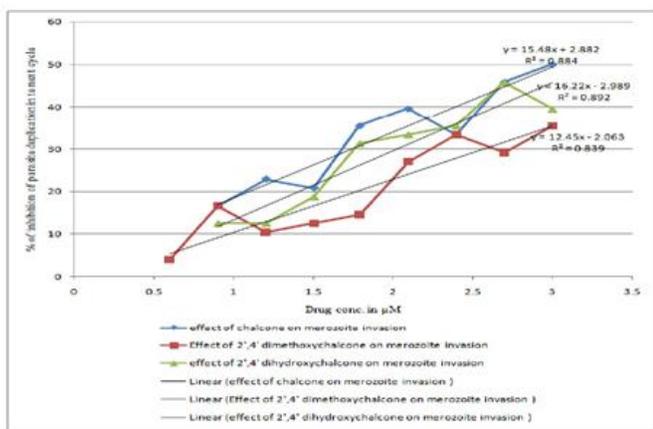
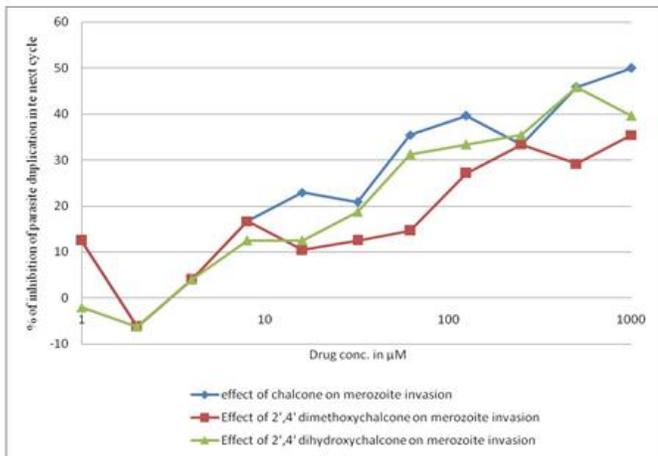


Fig 3 Percentage of inhibition of parasite duplication in the next cycle as a parameter of merozoite invasion versus drug concentration. Figure B shows the curve fitting of the log dose response curve

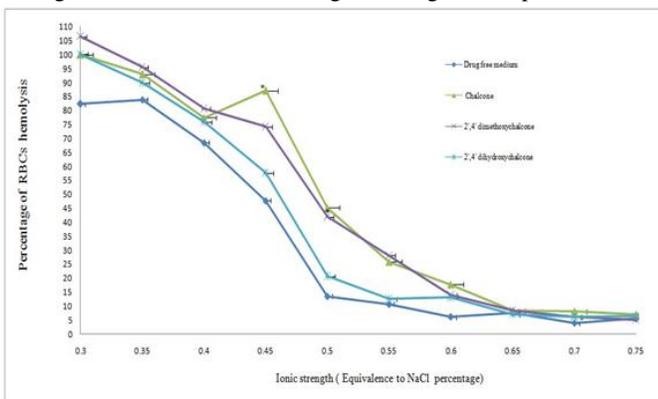


Fig 4 RBCs osmotic fragility curve wherein the ionic strength of the medium was represented as equivalence to NaCl concentration was plotted against percentage of RBCs hemolysis. The curve was plotted both in chalcones containing and free media. Each chalcone was at a concentration of 250 µM. The effect of each chalcone on the threshold ionic strength below which RBCs hemolysis is augmented was depicted. * refers to the statistical significance (P<0.05) of the hemolysis in the presence of the chalcone as compared to that in its absence.

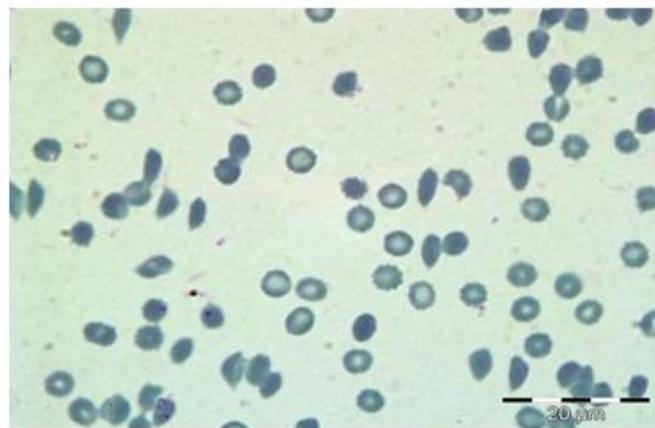


Fig 5:- RBCs morphology after exposure to the lipophilic chalcones. The figure shows some RBCs are transformed to echinocytes or spherocytes after exposure to chalcone

TABLE I

In vitro cytotoxic effect of each test compound on RBCs, Vero cells and plasmodium cell along with the selectivity index toward the plasmodium as compared to the both abovementioned cells. The last column represents the IC₅₀ values for the inhibitory action of the tested chalcones on NPPs

| Compound | IC ₅₀ against <i>Plasmodium falciparum</i> | IC ₅₀ against nRBCs stability | SI to plasmodium as compared to RBCs | IC ₅₀ against Vero cells stability | SI to plasmodium as compared to Vero | IC ₅₀ of drug induced inhibition of New Permeation Pathway |
|--------------------------------|---|--|--------------------------------------|---|--------------------------------------|---|
| Control | | | | | | |
| CQ | 255 nM | N.A | | N.A | | N.A |
| Chalcone | 38 µM | 280 µM | 7.6 | 522µM | 13.7 | 26 µM |
| 2',4'-dimethoxychalcone | 44 µM | 667 µM | 15 | 967µM | 22 | 8 µM |
| 2',4'-dihydroxychalcone | 86 µM | 801 µM | 9.8 | N.A | N.A | 10 µM |
| 4' chlorochalcone | 28.2 µM | 82 µM | 2.9 | 122 µM | 4.35 | - |
| 4-isopropyl-4'-methylchalcone | 33 µM | 43µM | 1.3 | 136 µM | 7.15 | - |
| 4-methyl-4'-methylthiochalcone | 12 µM | 88µM | 7.33 | 105 µM | 8.75 | - |

TABLE II

FIC₅₀ & FIC₉₀ based Isobologram analysis of different chalcones with chloroquine at different mixing proportions. Add., Indif., Syn. and Ant. mean additive, indifferent, synergistic and antagonistic actions respectively

| Chalcon | Combined preparation CQ/ chalcon | CQ mean FIC ₅₀ | Test chalcon FIC ₅₀ | FIC interaction | Interaction type | CQ mean FIC ₅₀ | Test chalcon FIC ₅₀ | FIC interaction | Interaction type |
|-------------------------|----------------------------------|---------------------------|--------------------------------|-----------------|------------------|---------------------------|--------------------------------|-----------------|------------------|
| Chalcon | 7:3 | 0.70 | 0.30 | 1.01 | Add | 0.69 | 0.18 | 0.87 | Add |
| | 5:5 | 0.67 | 0.67 | 1.34 | Indif | 0.82 | 0.94 | 1.76 | Indif |
| | 3:7 | 0.58 | 1.35 | 1.93 | Indif | 0.57 | 0.94 | 1.52 | Indif |
| 2',4'-dimethoxy-chalcon | 7:3 | 0.57 | 0.25 | 0.82 | Add | 0.72 | 0.17 | 0.89 | Add |
| | 5:5 | 0.61 | 0.64 | 1.24 | Indif | 0.63 | 0.32 | 0.98 | Add |
| | 3:7 | 0.41 | 1.00 | 1.41 | Indif | 0.51 | 0.67 | 1.19 | Indif |
| 2',4' dihydroxy-chalcon | 7:3 | 0.88 | 0.38 | 1.26 | Indif | 1.12 | 0.30 | 1.41 | Indif |
| | 5:5 | 0.73 | 0.75 | 1.48 | Indif | 0.94 | 0.47 | 1.41 | Indif |
| | 3:7 | 0.43 | 1.01 | 1.44 | Indif | 0.33 | 0.47 | 0.80 | Add |

TABLE IV

Results of the antioxidant activity of different chalcones

| Compound | Hydrogen peroxide Reducing IC ₅₀ | Reducing power | DPPH scavenging assay |
|--------------------------------|---|----------------|-----------------------|
| Chalcon | N.A | N.A | N.A |
| 2',4'-dimethoxychalcon | 135 µg/ml | 266 µg/ml | 125 |
| 2',4'-di-hydroxychalcon | 93 µg/ml | 102 µg/ml | 52 |
| 4' chloro-chalcon | N.A | N.A | N.A |
| 4-isopropyl-4' methylchalcon | N.A | N.A | N.A |
| 4-methyl-4'-methylthiochalcon | N.A | N.A | N.A |
| Butylated hydroxyl toluene BHT | | 43 µg/ml | 43 |
| Vitamin C | 450 µg/ml | 89 µg/ml | 96 |

TABLE 3 Physicochemical properties and the predictive bioactivity of the tested chalcones against four main intracellular targets; GPCR, ion channels, kinase, protease and nuclear receptors. The physicochemical parameters include ClogP, PSA, nON, nOHNH and nrotb. ClogP represents a global measure of the molecules hydrophilicity. PSA (polar surface area) is a measure of the surface area where the polarity is high. Furthermore, it is a measure of the sum of fragments contribution in conferring the polarity. natoms is the total number of atoms within the molecule, MW represents the molecular weight in gm/mole, nON is the total number of non hydrogen attached groups, nOHNH total number of hydrogen donating groups and nrotb is the total number of rotatable bond. The last is a measure of the molecules flexibility and is represented by single non ring bonds which are bounded to non terminal heavy atom (all except hydrogen). On the other hand, the predictive bioactivity parameters include the drug likeliness score which

is a measure of the coincidence of each compound with the standard inhibitors of each of GPCR, kinase and protease enzymes and the nuclear receptors as well AS TO THE standard ion channels modulator.

| Parameter | 4-methoxy-4'-methoxychalcon | 4-isopropyl-4'-methylchalcon | Chalcon | 2',4'-dihydroxychalcon | 2',4'-dimethoxychalcon | 4-chlorochalcon |
|----------------------------------|-----------------------------|------------------------------|---------|------------------------|------------------------|-----------------|
| Physiochemical properties | | | | | | |
| ClogP | 4.146 | 5.772 | 3.264 | 2.701 | 3.306 | 4.489 |
| PSA | 17.07 | 17.07 | 17.07 | 57.53 | 35.54 | 17.07 |
| atoms | 18.0 | 20.0 | 15.0 | 17.0 | 19.0 | 17.0 |
| MW (g/mole) | 256.3 | 264.3 | 196.2 | 228.2 | 256.3 | 242.7 |
| nON | 1 | 1 | 1 | 3 | 3 | 1 |
| nOHNH | 0 | 0 | 0 | 2 | 0 | 0 |
| nrotb | 4 | 4 | 3 | 3 | 5 | 3 |
| Predictive bioactivity | | | | | | |
| GPCR ligand | | -0.21 | -0.43 | -0.17 | -0.15 | -0.35 |
| Ion channel modulator | -0.30 | -0.21 | -0.18 | -0.09 | -0.21 | -0.16 |
| Kinase inhibitor | -0.61 | -0.43 | -0.66 | -0.42 | -0.40 | -0.58 |
| Nuclear receptor ligand | -0.32 | -0.16 | -0.51 | -0.05 | -0.13 | -0.45 |
| Protease inhibitor | -0.33 | -0.37 | -0.60 | -0.29 | -0.26 | -0.57 |
| Enzyme inhibitor | -0.17 | -0.08 | -0.12 | 0.06 | -0.05 | -0.13 |

REFERENCES

- [1] Overbosch, D.v.d.W.B., AW Stuijver, PC van der Kaay, HJ: Chloroquine-resistant falciparum malaria from Malawi. Trop Geogr Med, 1984. 36: p. 71 - 72.
- [2] Ponnudurai, T., A. Leeuwenberg, and J. Meuwissen, Chloroquine sensitivity of isolates of Plasmodium falciparum adapted to in vitro culture. Trop Geogr Med, 1981. 33: p. 50 - 54.
- [3] Trape, J., The public health impact of chloroquine resistance in Africa. Am J Trop Med Hyg, 2000.64(suppl): p. 12 - 17.
- [4] Witkowski B, B.A.B.-V.F., Resistance to antimalarial compounds: Methods and applications. Drug Resist Updat, 2009. 12: p. 42 - 50. <http://dx.doi.org/10.1016/j.drug.2009.01.001>
- [5] Stephanie G. V., J.-C.V., Lise M, Lisa A , Odile M et al Identification of a Mutant PfCRT- Mediated Chloroquine Tolerance Phenotype in Plasmodium falciparum. Plos pathogens 2010.6(5): p. e1000887.
- [6] Maud Henry, S.A., Eve Orlandi-Pradines, Hervé Bogreau, Thierry Fusai, Christophe Rogier, Jacques arbe and Bruno Pradines, Chloroquine Resistance Reversal Agents as Promising Antimalarial Drugs. Current Drug Targets, 2006. 7: p. 935-948. <http://dx.doi.org/10.2174/138945006778019372>
- [7] Batovska DI, T.I., Trends in utilization of the pharmacological potential of chalcones. Curr Clin Pharmacol. , 2010. 5(1): p. 1-25. <http://dx.doi.org/10.2174/157488410790410579>
- [8] Opletalová, V., Chalcones and their heterocyclic analogs as potential therapeutic agents in bacterial diseases. Ceska Slovenska Farmacie, 2000. 49(6): p. 278-84.
- [9] Quinton L. Fivelman, I.S.A., and David C. Warhurst, Modified fixed-ratio isobologram method for studying In vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of Plasmodium falciparum. Antimicrobial Agents and Chemotherapy, 2004.48(11): p. 4097-4102. <http://dx.doi.org/10.1128/AAC.48.11.4097-4102.2004>
- [10] Cranmer SL Magowan C, L.J., Coppel RL, Cooke BM., An alternative to serum for cultivation of Plasmodium falciparum in vitro. . Trans R Soc Trop Med Hyg, 1997. 91(3): p. 363-365. [http://dx.doi.org/10.1016/S0035-9203\(97\)90110-3](http://dx.doi.org/10.1016/S0035-9203(97)90110-3)
- [11] Trager W, J.J.B., Human malaria parasites in continuous culture. Science, 1976. 193: p. 673-5. <http://dx.doi.org/10.1126/science.781840>
- [12] VanderbergJP., L.a., Synchronization of Plasmodium falciparum erythrocytic stages in culture. Journal of parasitology 1979. 65(3): p. 418-420.

- <http://dx.doi.org/10.2307/3280287>
- [13] Matthias G. Vossen , S.P., Peter Chiba , and Harald Noedl The SYBR Green I Malaria Drug Sensitivity Assay: Performance in Low Parasitemia Samples. *The American Society of Tropical Medicine and Hygiene*, 2010. 83(3): p. 389-401.
- [14] Van Meerloo J, K.G., Cloos J., Cell sensitivity assays: the MTT assay. *Methods Mol Biol.*2011. 731: p. 237-45.
http://dx.doi.org/10.1007/978-1-61779-080-5_20
- [15] Mary Luz López, S.B., Jairo Sáez & Cesar Segura, Effect of Solanum nudum steroids on uninfected and Plasmodium falciparum-infected erythrocytes. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, 2009. 104(5): p. 683-688.
<http://dx.doi.org/10.1590/S0074-02762009000500003>
- [16] Go ML, L.M., Wilairat P, Rosenthal PJ, Saliba KJ, Kirk K. , Antiplasmodial chalcones inhibit sorbitol-induced hemolysis of Plasmodium falciparum-infected erythrocytes. *Antimicrobial Agents & Chemotherapy*, 2004: p. 3241-3245.
<http://dx.doi.org/10.1128/AAC.48.9.3241-3245.2004>
- [17] Staines HM, D.B., O'Brien M, Lang HJ, Englert H, Horner HA, Ellory JC, Kirk K., Furosemide analogues as potent inhibitors of the new permeability pathways of Plasmodium falciparum- infected human erythrocytes. *Mol Biochem Parasitol*, 2004. 133(2): p. 315-8.
<http://dx.doi.org/10.1016/j.molbiopara.2003.10.009>
- [18] Ruch, R.J., Cheng, S.J., and Klaunig, J.E. , Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 1989. 10: p. 1003-1008.
<http://dx.doi.org/10.1093/carcin/10.6.1003>
- [19] Oyaizu, M., Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, 1986. 44: p. 307-315.
<http://dx.doi.org/10.5264/eiyogakuzashi.44.307>
- [20] Hatano, T., Kagawa, H., Yasuhara, T., & Okuda, T. , wo new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *T Chemical and Pharmaceutical Bulletin de la Societe de Pathologie Exotique*, 1988. 36: p. 2090-2097.
- [21] Gerardo Cebrian-Torrejón, K.S., Karine Leblanc; Katalina Muñoz-Durango, Sandra Torijano Gutierrez. , The antiplasmodium effects of a traditional South American remedy: Zanthoxylum chiloperone var. angustifolium against chloroquine resistant and chloroquine sensitive strains of Plasmodium falciparum. 2011. 21: p. 4.
- [22] Nowakowska Z., A review of anti-infective and anti-inflammatory chalcones. *European Journal of Medicinal Chemistry*, 2007. 42(2): p. 125-37.
<http://dx.doi.org/10.1016/j.ejmech.2006.09.019>
- [23] Belsare D .P, P.S.C., Kazi A .A., Kankate R.S4., Vanjari S.S, Evaluation of antioxidant activity of chalcones and flavonoids. *International Journal of ChemTech research* 2010. 2(2): p. 1080-1089.
- [24] Librado A. Santiago, A.B.R.M., Prooxidant Effect of the Crude Ethanolic Leaf Extract of Ficus odorata Blanco Merr. in vitro: It's Medical Significance. *International Journal of Biological, Veterinary, Agricultural and Food Engineering*, 2014. 8(1): p. 53-60.
- [25] Nemeikaitė-Čėnienė, A., et al. , Quantitative structure-activity relationships in prooxidant cytotoxicity of polyphenols: Role of potential of phenoxyl radical/phenol redox couple. *Archives of Biochemistry and Biophysics*, 2005. 441(2).
<http://dx.doi.org/10.1016/j.abb.2005.07.002>
- [26] Mozuraityte, R., et al. , Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants. *J. Agric. Food Chem.*, 2009. 57(21): p. 10377-10385.
<http://dx.doi.org/10.1021/jf901072t>
- [27] Maurya, D.a.D., T., Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids. *Food Chem. Toxicol*, 2010. 48: p. 3369-3373.
<http://dx.doi.org/10.1016/j.fct.2010.09.006>
- [28] Go Mei-Lin, L.M., Wilairat Prapon, Rosenthal Philip J., Saliba Kevin J., Kirk Kiaran, Antiplasmodial Chalcones Inhibit Sorbitol-Induced Hemolysis of Plasmodium falciparum- Infected Erythrocytes. *Antimicrobial Agents and Chemotherapy*, 2004. 48(9): p. 3241-3245.
<http://dx.doi.org/10.1128/AAC.48.9.3241-3245.2004>
- [29] Go Mei-Lin, L.M., Wilairat Prapon, Rosenthal Philip J., Saliba Kevin J., Kirk Kiaran, Antiplasmodial Chalcones Inhibit Sorbitol-Induced Hemolysis of Plasmodium falciparum- Infected Erythrocytes. *Antimicrobial Agents and Chemotherapy*, 2004. 48(9): p. 3241-3245.
<http://dx.doi.org/10.1128/AAC.48.9.3241-3245.2004>
- [30] Silfen J, Y.P., Cabantchik ZI., Bioflavonoid effects on in vitro cultures of Plasmodium falciparum. Inhibition of permeation pathways induced in the host cell membrane by the intraerythrocytic parasite. *Biochemistry and pharmacology*, 1988. 37(22): p. 4269-76.
[http://dx.doi.org/10.1016/0006-2952\(88\)90606-5](http://dx.doi.org/10.1016/0006-2952(88)90606-5)
- [31] Silfen J, Y.P., Cabantchik ZI., Bioflavonoid effects on in vitro cultures of Plasmodium falciparum. Inhibition of permeation pathways induced in the host cell membrane by the intraerythrocytic parasite. *Biochemistry and pharmacology*, 1988. 37(22): p. 4269-76.
[http://dx.doi.org/10.1016/0006-2952\(88\)90606-5](http://dx.doi.org/10.1016/0006-2952(88)90606-5)
- [32] Laura M. Sanz, B.C., Cristina De-Cózar, Xavier C. Ding, Jose L. Llergo, Jeremy N.Burrows, Jose F. Garcí'a-Bustos, Francisco-Javier Gamó, P. falciparum In Vitro Killing Rates Allow to Discriminate between Different Antimalarial Mode-of-Action. *Plos one*, 2012. 7(2): p. e30949.
<http://dx.doi.org/10.1371/journal.pone.0030949>
- [33] Witkowski, B., J. Lelièvre, et al. , Increased Tolerance to Artemisinin in Plasmodium falciparum Is Mediated by a Quiescence Mechanism. *Antimicrob. agent Chemother.*, 2010. 54(5): p. 1872-1877.
<http://dx.doi.org/10.1128/AAC.01636-09>
- [34] Ette EI, E.E., Thomas WO, Brown-Awala EA., Pharmacokinetics of chloroquine and some of its meTABLEolites in healthy volunteers: a single dose study. *Journal of clinical pharmacology* 1989,29(5): p. 457-62.
<http://dx.doi.org/10.1002/j.1552-4604.1989.tb03362.x>