

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

Zaid OI<sup>1</sup>, Abd Majid R<sup>2</sup>, Basir R<sup>3</sup>

**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, it's 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

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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).

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### *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, 20 µg/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% N<sub>2</sub>, 5% CO<sub>2</sub> and 5% O<sub>2</sub>. 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 (Vanderberg 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 100 µL (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 IC<sub>50</sub> and IC<sub>90</sub>*

Both IC<sub>50</sub> and IC<sub>90</sub> 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 IC<sub>50</sub>. The dilution was chosen such that IC<sub>50</sub> 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 IC<sub>50</sub> and IC<sub>90</sub> of each combination separately. For each combination ratio, both FIC<sub>50</sub> and FIC<sub>90</sub> (fractional inhibitory concentration) were calculated through finding the ratio of IC<sub>50</sub> or IC<sub>90</sub> 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, FIC<sub>50</sub> values for CQ and each chalcon were extrapolated on Y and X axes respectively. Similarly, FIC<sub>90</sub> 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 thorough 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 (VersaMax<sup>tm</sup>). 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% CO<sub>2</sub>, 5% O<sub>2</sub> and 90 % N<sub>2</sub> 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 IC<sub>50</sub> against Vero cells was calculated using Microsoft excel 2007.

#### *Selectivity index*

Selectivity index was calculated through finding the ratio of the drug IC<sub>50</sub> 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 thorough 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, IC<sub>50</sub> (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.

#### *Physiochemical properties calculation and bioactivity prediction*

Both physiochemical 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 (IC<sub>50</sub>= 265 ± 3.3 µM, IC<sub>90</sub>= 433 nM). It was poorly responsive to all the mentioned chalcones with IC<sub>50</sub> 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 IC<sub>50</sub> 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 IC<sub>50</sub> 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).

#### *Physiochemical properties and bioactivity prediction*

Results of Molinspiration software ([www.molinspiration.com](http://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-likeness 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 methoxylated 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 IC<sub>50</sub>s 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 IC<sub>50</sub> 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 IC<sub>50</sub> 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 IC<sub>50</sub> for the drug effect on NPPs approaches to 10  $\mu$ M. 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 IC<sub>50</sub> and IC<sub>90</sub> 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 IC<sub>90</sub> rather than affecting the IC<sub>50</sub>.

Antagonism was absent in all the combinations as none of them produced a sum for FIC<sub>50</sub>tot or FIC<sub>90</sub> > 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 IC<sub>90</sub> based isobologram rather than the IC<sub>50</sub> 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 IC<sub>50</sub> 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 IC<sub>90</sub> 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 IC<sub>50</sub>s 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.

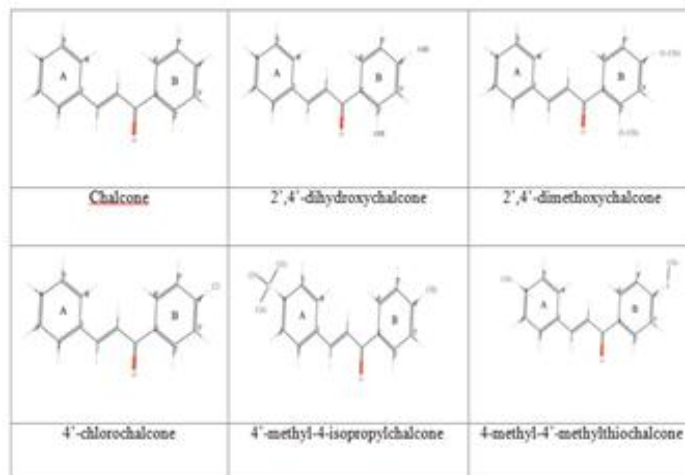


Fig 1 :- Chemical structures of the chalcone and the chalcon derivatives that was used in the study. The structure in the upper left corner represents the structure of the basic chalcon nucleus that is made of a di-aryl keton structure (1,3-Diphenyl-2-propen-1-one). It contains two rings; A & B. Any substitution on ring B is tagged by “'”. Substitutions on ring B 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.

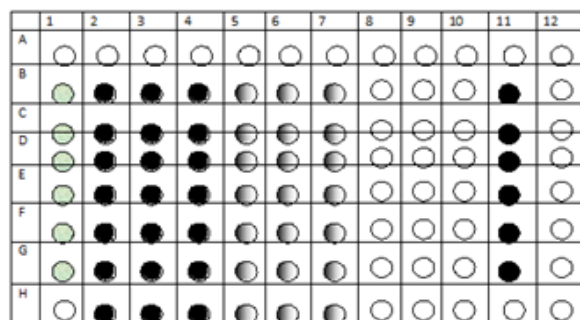


Fig 2:- Isobologram layout on flat bottomed 96 well plate with concentration ratios of CQ to each of the test phytochemical as three solutions (with CQ/phytochemical ratios 7:3, 5:5 and 3:7 respectively). When the plates were prepared as described in the text, wells tagged with serve as an RBC control (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

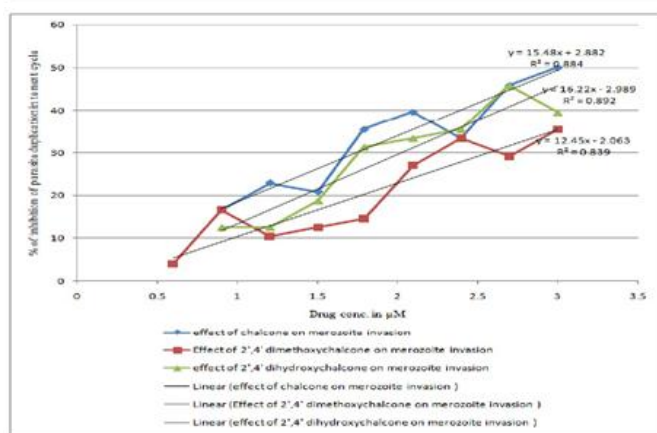
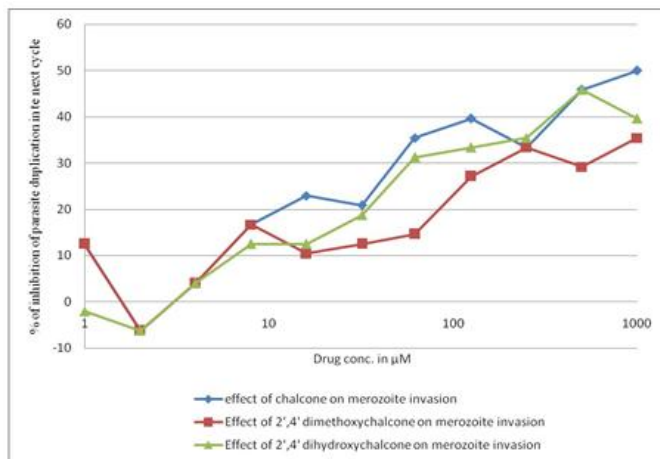


Fig 3 Percentage of inhibition of parasite duplication in the next cycle as a parameter of merozoite invasion versus drug concentration.

Figure B shows the curve fitting of the log dose response curve

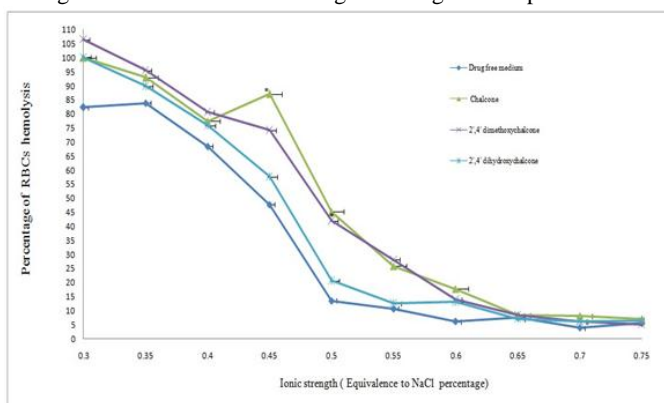


Fig 4 RBCs osmotic fragility curve wherein the ionic strength of the medium was represented as equivalence to NaCl concentration was plotted against percentage of RBCs hemolysis. The curve was plotted both in chalcones containing and free media. Each chalcone was at a concentration of 250 µM. The effect 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.

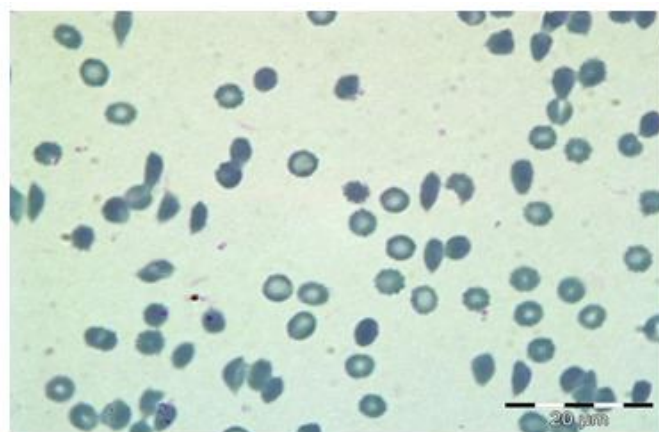


Fig 5:- RBCs morphology after exposure to the lipophilic chalcones. The figure shows some RBCs are transformed to echinocytes or spherocytes after exposure to chalcone

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  $IC_{50}$  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	522 µM	13.7	26 µM
2',4'-dimethoxychalcone	44 µM	667 µM	15	967 µM	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	43 µM	1.3	136 µM	7.15	-
4-methyl-4'-methylthiochalcone	12 µM	88 µM	7.33	105 µM	8.75	-



TABLE II

FIC<sub>50</sub> & FIC<sub>90</sub> 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 chalcon CQ/ preparation	CQ mean FIC <sub>50</sub>	Test chalcon FIC <sub>50</sub>	FIC interaction	Interaction type	CQ mean FIC <sub>50</sub>	Test chalcon FIC <sub>50</sub>	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 <sub>50</sub>	Reducing power	DPPH scaveng ing 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 Physiochemical properties and the predictive bioactivity of the tested chalcones against four main intracellular targets; GPCR, ion channels, kinase, protease and nuclear receptors. The physiochemical 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-methoxyl-4'-methoxythiochalcon	4-isopropyl-4'-methoxychalcon	Chalcon	2',4'- dihydroxychalcon	2',4'- dimethoxychalcon	4-chlorochalcon
<b>Physiochemical properties</b>						
<b>ClogP</b>	4.146	5.772	3.264	2.701	3.306	4.489
<b>PSA</b>	17.07	17.07	17.07	57.53	35.54	17.07
<b>natoms</b>	18.0	20.0	15.0	17.0	19.0	17.0
<b>MW (g/mole)</b>	256.3	264.3	196.2	228.2	256.3	242.7
<b>nON</b>	1	1	1	3	3	1
<b>nOHNH</b>	0	0	0	2	0	0
<b>nroth</b>	4	4	3	3	5	3
<b>Predictive bioactivity</b>						
<b>GPCR ligand</b>		-0.21	-0.43	-0.17	-0.15	-0.35
<b>Ion channel modulator</b>	-0.30	-0.21	-0.18	-0.09	-0.21	-0.16
<b>Kinase inhibitor</b>	-0.61	-0.43	-0.66	-0.42	-0.40	-0.58
<b>Nuclear receptor ligand</b>	-0.32	-0.16	-0.51	-0.05	-0.13	-0.45
<b>Protease inhibitor</b>	-0.33	-0.37	-0.60	-0.29	-0.26	-0.57
<b>Enzyme inhibitor</b>	-0.17	-0.08	-0.12	0.06	-0.05	-0.13

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