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Antiplasmodial and chloroquine chemosensitizing and resistance reversal effects of coumarin derivatives against *Plasmodium falciparum* 3D7 and K1

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Abstract. **Background** Emergence of **c**hloroquine (CQ) resistance among different strains of *Plasmodium falciparum* is the worst incident that has ever faced the dedicated efforts to eradicate malaria. The main cause of CQ resistance is over-activity of the pumping mechanism that ousts CQ outside the DV. This urged the scientists to look for other alternatives or adjuvants that augment its action. CQ The study aimed to test the potential of five coumarin derivatives, namely; umbeliferon, esculetin, scopoletine, herniarin and 3-aminocoumarine to inhibit plasmodium growth and reverse CQ resistance in *Plasmodium falciparum* K1 and 3D7. They are highly ubiquitous in nature and are famous by their diverse pharmacological effects. SYBRE green-1 based drug sensitivity assay was used to screen the effect of CQ and each coumarin on the parasite growth and isobologram technique was to assess the interaction of the coumarins with CQ. Effect of each coumarin on both RBCs and Vero cells stability as well as on RBCs fragility were screened to exclude any toxic impact on normal cells. On the other hand, their effect on hemozoin formation was screened to investigate about their molecular mechanism. For molecular characterization, Their antioxidant properties were determined using the conventional *in vitro* tests and their characters were obtained from Molinspiration Simulation Software. Results showed that all of them were safe to human cells, have weak to moderate plasmodial growth inhibitory effect and only umbeliferon, 3-aminocoumarin and esculetin has interacted effectively with CQ. These actions are neither correlated with hemozoin formation inhibition nor to the antioxidant mechanisms. Further studies recommended to investigate the mechanism of their action. Overall, all the tested coumarins are not ideal to be used in the conventional malaria therapy and only umbeliferon, 3-aminocoumarin and esculetin can be suggested to potentiate CQ action.

INTRODUCTION

In spite of the achieved progress in parasitic ailments eradication, malaria is still a major therapeutic challenge in the developing countries due to emergence of drug resistance and tolerance among different strains of *Plasmodium falciparum* (Overbosch 1984). Loss of Chloroquine (CQ) activity is the worst incident have ever faced the dedicated efforts to eradicate malaria as it is still the most pertinent anti-malarial due to its relative safety, cost affectivity and efficiency in comparison to other

conventional anti malarials (Trape 2000) (Witkowski B 2009) (Witkowski B Lelie'vre J 2012) and (Stephanie G.V. 2010). Drug resistance reduces their response to higher doses while appearance of the tolerance increases the prevalence of the disease re-occurrence (Stephanie G.V. 2010). This issue has urged the scientist to search for other alternatives or chemo-sensitizers that potentiate CQ action (Maud Henry 2006).

Over the recent past, many modern medicines have been derived from natural products, viz; artimisinin; a natural compound obtained from the shrub *Artemisia annua*. On the other hand, lots of phytochemicals were found to synergize CQ Some of them could have performed this in spite of their the absence of any intrinsic *in vitro* and *in vivo* growth inhibitory effects on plasmodium (H Rafatro 2000), (M Frédérich 2001) & (P Rasoanaivo 1996). These studies have opened the door for the trials to implement such compounds as chemo-sensitizers for CQ. In this experiment, the potential of five coumarine derivatives, namely; esculetin, scopoletine, herniarin, umbeliferon and 3-aminocoumarine to interfere with growth and reverse CQ resistance in *Plasmodium falciparum* was studied.

Coumarins are benzo-á-pyron derivatives (compounds containing aromatic ring fused with lactone moiety (Figure 1). Their dietary exposure is quite frequent due to their abundant distribution in natural food products. They are subdivided into simple, furano and pyrono coumarins. All the chosen coumarins in our study belong to the first class. Previous studies has pointed out to their significant anti-tumor, anti-microbial, anti-oxidant, anti-inflammatory and anti-edematous effects (O'Kennedy 2004).

Different compounds act on different chemotherapeutic targets, viz; malaria parasite protease enzyme, detoxification of heme, inhibition of some cytoplasmic targets like fatty acid or isoprenoid synthesis pathways, histidin rich protein or plasmodial protein kinase (Olliaro PL 1999). Meanwhile, CQ synergism may be conferred by drugs that augment its effect on the digestive vacuole (DV), enhance the intra-vacuolar CQ accumulation, promote DV membrane permeability and subsequent seeping of the hydrolytic enzymes or augment the CQ induced apoptotic pathway (OlliaroP 2001) and (J-H Ch'ng 2011).

CQ induced death occurs due to inhibition of heme detoxification that results in damaging the DV membrane, releasing the hydrolytic enzymes and subsequent autolysis of the plasmodium protoplasm. Recently, it has been found that induction of the DV membrane permeabilization is another mechanism through which CQ induces cellular degeneration at concentrations lower that what is required to hinder hemozoin formation. DV membrane permeabilization induces seeping of the low g.m.wt hydrolytic enzymes, viz; cathepsin; which triggers the sequential cascade of apoptosis induction (J-H Ch'ng 2011).

MATERIALS AND METHOD

2.1 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 form Sigma-Aldrich (St. Louis, MO, USA). Gentamicin was purchased from Jiangxi

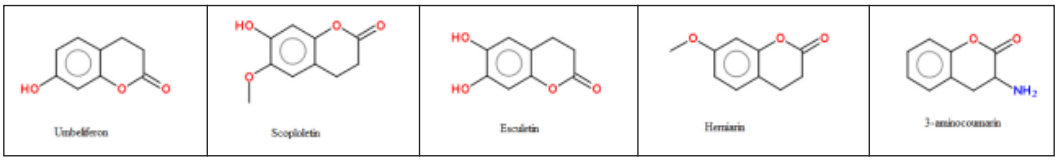

Figure 1. Chemical structure of 5 coumain derivatives, namely; umbeliferon, scopoletin, esculetin, herniarin and 3-aminocoumarin.

Dongxu Chemical Technology Co., Ltd. umbeliferon, esculetin, scopoletin, herniarin and 3-aminocoumarin (Figure 1) were purchased from Indofine Biochemical Company Inc. (Cat. No.: H-017, D-108, H-109, H-101and A-104 respectively).

2.2 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 gentamicine and 25 mM HEPES). It was kept in the fridge overnight and used for not more than one week after its preparation (Cranmer SL Magowan C 1997) .

2.3 Parasite culturing, maintenance and synchronization

2.3.a – Parasite maintenance

Plasmodium falciparum K1and 3D7 (ATCC), procured from the Institute of Medical Research, Kuala Lumpur Malaysis, 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-ethan-sulphonic acid) buffer (pH 7.4), 0.75 mM hypoxanthine, 0.5% albumax, 24 mM sodium bicarbonate, 11 mM glucose and 50 ìg/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_2, 5% CO_2 and 5% O_2. The medium was changed every 24 hrs and the growth was checked using Giemsa stained thin blood smears (Trager 1987) & (Cranmer SL Magowan C 1997).

2.3.b – 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 (section 2.2). (Vanderberg JP. 1979)

2.4 Stock solution preparation

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

2.5 Malaria drug sensitivity assay

Malaria drug sensitivity assay was performed as previously described by Mathias *et al.*, 2010. (Matthias G. Vossen 2010). Both IC_{50} and IC_{90} of each test drug against *Plasmodium falciparum* K1 were determined using Microsoft excel 2007 software after plotting the curve of percentage of parasite inhibition versus log [drug concentration].

2.6 Drug combination assay and isobologram analysis

Interaction of each coumarin with CQ was screened using the isobologram technique described by (Quinton L. Fivelman 2004). Briefly, working solutions of each drug were prepared at 16 times their IC_{50} values and were mixed at different ratio ranging from 10:0 to 0:10; ratios of CQ/ phytochemical). Aftermath, each combination was serially diluted and each dilution was mixed with plasmodium infected RBCs for 48 hrs at the standard conditions. Finally IC_{50} and IC_{90} of each combination were determined separately and both FIC_{50} and FIC_{90} (fractional inhibitory concentration) were calculated from the ratio of the drug's IC_{50} or IC_{90} within the combination to those when the drug was incubated with the parasite alone (Quinton L. Fivelman 2004). These values were used to plot the isobologram as described by (Quinton L. Fivelman 2004) which gives a clear picture for the type of the interaction.

2.7 Effect on non infected RBCs

2.7.1 Effect of on RBCs stability

Different concentrations of each coumarin (1 nM - 1 mM) were incubated with O +ve human RBCs in an Incomplete **C**ulture **M**edium **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, the RBCs were washed out and the supernatant from each well was 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.

2.7.2 Effect on RBCs fragility

RBC fragility test was performed as previously described (Ismail Karabulut 2009). Briefly, washed RBCs were suspended in series of solutions of different tonicity ranges 0-0.9% noticing that 0% tonicity gives 100% hemolysis and incubated with three different concentrations of each coumarin; 1µM, 500 µM and 1m. Finally, percentage of hemolysis vs. tonicity curve was extrapolated for each drug concentration and was compared to that of the drug free set.

2.8 Effect of the phytochemicals Vero cells

Vero cells were incubated for 48 hrs with different concentrations of each compound (1 nM-1mM) at 37ºC, 5% CO_2, 5% O_2 and 90% N_2 in a culture medium containing RPMI-1640, 10% BSA (Bovin serum albumin) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). After incubation, MTT assay was performed as previously described (Carstens 2004) (Van Meerloo J 2011).

2.9 Effect of on merozoites invasiveness

Effect on merozoite invasion was performed as previously described (Lazarou 2009). Briefly, non-infected RBCs were treated for 2 hrs. with different concentrations of the drug (1 µM-1 mM) dissolved in the Incomplete Malaria Culture Medium. 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. Then the amount of merozoites that could have invaded the treated RBCs was compared to the positive control that contains only non-treated RBCs.

2.10 Molecular characters assessment

2.10.1 Antioxidant activity

The antioxidant activity of each coumarin was screened as described previously using hydrogen peroxide scavenging (Ruch 1989), reducing power (Oyaizu 1986) and DPPH scavenging activity assays (Hatano 1988).

2.10.2 Physiochemical properties calculation and bioactivity prediction

Both physiochemical properties and the predicted bioactivity parameters for each coumarin were determined using chemi-informatic software known as Molinspiration (http://www.molinspiration.com). It performs a fragment based virtual screening of the following parameters, 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.

2.10 Effect on hemozoin formation

2.10.1 β-hematin formation assay

The potential of each coumarin to hinder heme polymerization and hinder hemozoin formation was assayed using β-hematin formation inhibition assay (βHIA) as described by (Silvia Parapini 2000).

2.10.2 Drug heme interaction

Effect of each drug on Sorret band was monitored through scanning the absorbance of the UV-visible spectrum at wavelengths between 300-600 nm of heme-drug mixtures containing 50 µM of hemine chloride along with 0.5, 1, 5, 10, 25, 50 and 100 µM of each drug.

RESULTS

Drug sensitivity assay

In spite of the prominent difference in CQ sensitivity between *Plasmodium falciparum* K1 and 3D7, each coumarin produced a comparable effect on each. Nevertheless they are still considered weak as compared to CQ (Table 1).

Effect on CQ sensitivity

All the tested coumarins failed to produce any synergy with CQ except for esculetin when it was mixed at 7:3 (CQ/ drug) ratios while other combinations of the two dugs showed only an additive effect as seen in both IC_{50} & IC_{90} based isobolograms (Figure 3). Both umbeliferon and 3-aminocoumarin produced an additive effect when they were combined with CQ at a ratio of 7:3 (CQ/drug) in the two isobolograms. The rest compounds did not produce any interaction with CQ (Figure 3).

Effect on nRBCs and mammalian Vero cells stability, merozoit invasion and RBCs fragility

All the tested coumarines were patent to the RBCs and Vero cells ($IC_{50} > 1$ mM) and no any sign of damage or toxicity was obtained. They did not affect BCs fragility as the threshold ionic strength was similar in the drug treated and non-treated samples. Furthermore, they did not have any impact on merozoit invasion.

Physiochemical properties and bioactivity prediction

Anti-oxidant activities of each coumarin are illustrated in Table 3. All of them showed a potential to prevent the oxidative stress. The effect was more prominent in esculetin and umbeliferon and less for 3-aminocoumarin (Table 3).

Results of Molinspriation software (www.molinspiraton.com) are illustrated in (Table 4) wherein the physiochemical properties of the compounds are represented by numerical values. Its predictive bioactivity against the main intracellular targets; GPCR, kinase, protease and nuclear receptors as represented by drug-likeliness score.

Effect on β-hematin formation

All of the tested coumarins failed to inhibit β-hematin formation ($IC_{50} > 40$ mM) or slake down the Soret band intensity in heme binding assay (Figure 4) except for 3 aminocoumarin which showed weak inhibitory effect and binding capacity to heme moieties.

DISCUSSIONS

Previous studies had pointed out to the role of natural products in eradicating malaria parasite or reverse chloroquin CQ resistance in the resistant strains of *Plasmodium falciparum*. Few of them were worth to be used to treat malaria in the clinical field, such as; artimisia annue On the other hand, CQ resistance reversing effect was seen for some natural products in spite of the absence of both the in vivo and in vitro intrinsic anti-plasmodium effect.

Coumarins comprise a wide class of phytochemicals with a profound distribution in plant kingdom. They have diverse range of biological activities ranging from anti-tumor, anti-inflammatory, anti-microbial, anti-coagulant and anti-edematous effects. They are made up of an aromatic ring fused with pyran ring with substitution on the aromatic rings (O'Kennedy 2004). Results of the Molispiration software show that all of them have good hydrophilicity with ClogP less than 2. The molispiration simulation software predicts ubiquity of an effect of such coumarins on some specific intracellular targets, viz; GPCR (G protein coupled receptors), kinase and protease enzymes as well as nuclear receptors. Generally, all the substitutions have improved the drug likeliness score as compared to the parent coumarin nucleus (Table 2). Drug likeliness score is a complex balance of various molecular properties to determine the similarity of the test drugs with standard inhibitors. It is a magic number that expresses some characters, viz, hydrophobicity, electronic distribution, hydrogen bonding, molecular size or the pharmacophoric characters that entitle the compound to produce a pharmacological action. Nevertheless, according to the software

prediction, all of them are not strong inhibitors for the mentioned targets. The bioactivity predictive tool showed that 3-aminocoumarin, esculetin and scopletine exhibited the best drug-likeliness scores as compared to the others. Although, all of these actions were suggested on human cells and further studies are required to explore their ubiquity in the less developed parasite cells.

It is noteworthy that all the tested coumarins failed to produce any impact on red blood cells RBCs stability. This may be attributed to their relative poor lipophilicity that compromises its tendency to accumulate in cell membranes. Furthermore, they did not show any tendency to bind to heme moieties excluding any possibility for such compounds to interact with RBCs heme.

Table 1. Results of drug sensitivity assay for CQ, umbeliferon, scopoletin, esculetin, herniarin and 3-aminocoumarin on *Plasmodium falciparum* K1 and 3D7. IC_{50} and IC_{90} of all the coumarins in the micromolar while those of CQ were in the nano-molar range

	K1		3D7	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Chloroquine	265 ± 3.3 nM	433 ± 8.8 nM	17 ± 3.4 nM	36.3 ± 4.5 nM
Umbeliferon	138 ± 3.7 µM	353 ± 7.2 µM	131 ± 4.8 µM	362.1 ± 5.9 µM
Esculetin	113 ± 4.4 µM	476 ± 9.2 µM	129 ± 6.7 µM	444.2 ± 11.1 µM
Scopoletin	128 ± 2.7 µM	459.7 ± 2.6 µM	121.5 ± 3.6 µM	433.4 ± 5.7 µM
Herniarin	167.8 ± 6.4 µM	559.7 ± 7.2 µM	172.5 ± 5.7 µM	533.4 ± 6.1 µM
3-aminocoumarin	95.6 ± 3.2 µM	226 ± 4.3 µM	102.3 ± 4.2 µM	218.3 ± 5.3 µM

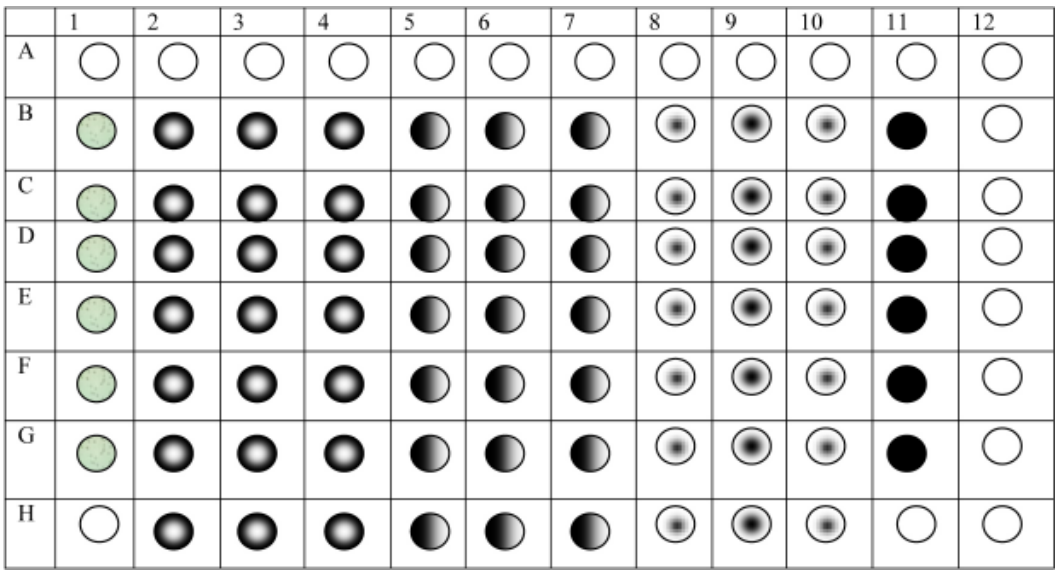

Figure 2. Isobologram layout on flat bottomed 96 well plate with concentration ratios of CQ to each of the test phytochemical as three solutions (with CQ/phytochemical ratios 7:3, 5:5 and 3:7 respectively. When the plates were prepared as described in the text, wells tagged with ◯ serve as an RBC control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

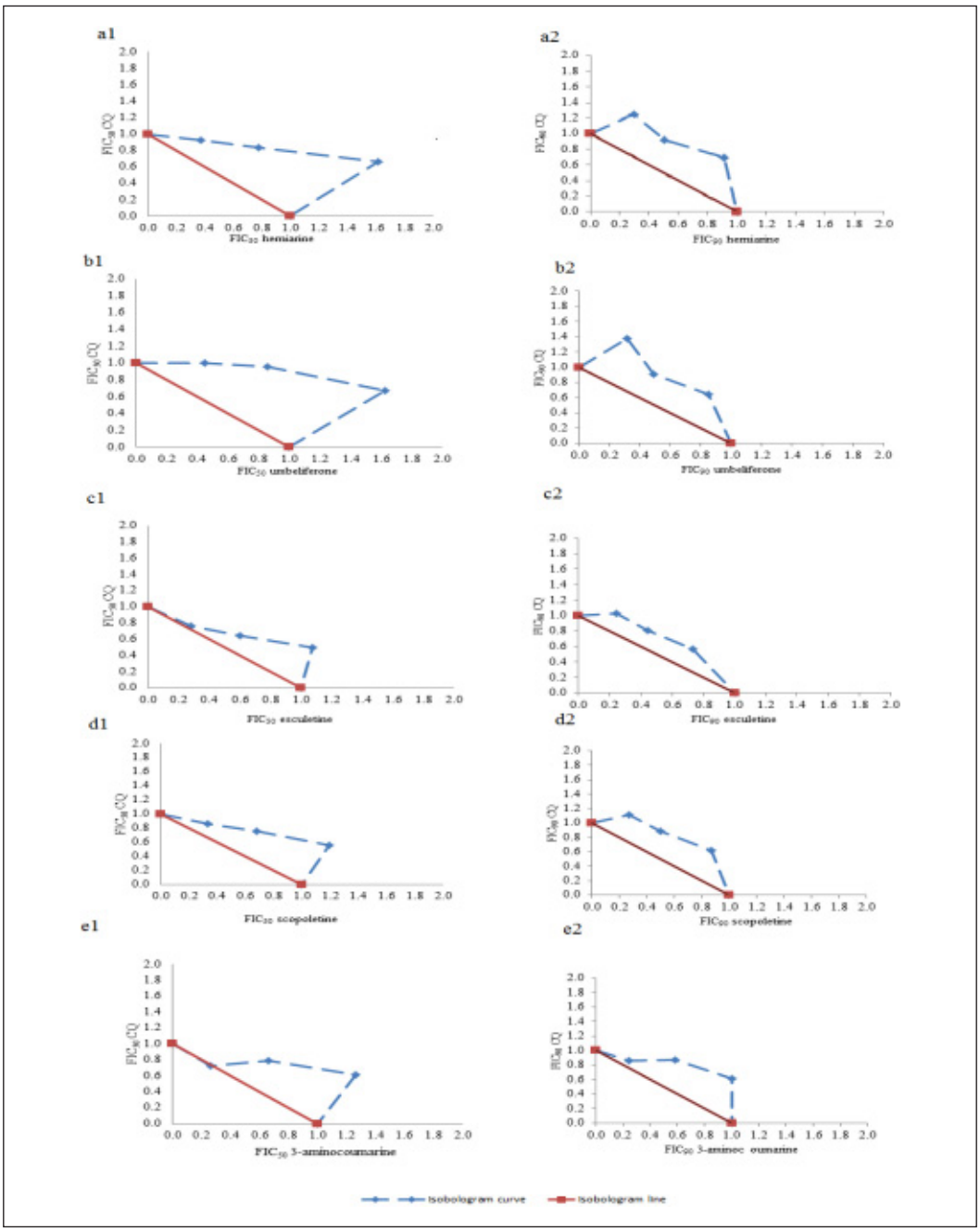

Figure 3. Isobologram curves based showing the interaction of CQ with five coumarin derivatives, namely; umbeliferon, herniarin, esculetin, scopoletin and 3- aminocoumarin. Two isobologram curves were plotted for each combination; FIC_{50} values for CQ and each coumarin were extrapolated on Y and X axes respectively and FIC_{90} was extrapolated instead of FIC_{50} in the second. The line that links the two drugs FICs is considered as line of additivity (solid line) such that, the interaction is considered additive when the points fall on the line or total FIC is equal to 1, synergism when they fall below the line of additivity and indifference and antagonism if they fall above that line respectively. Antagonism is considered only when the total FIC is >2 (Quinton L. Fivelman 2004). a, b and c represents both IC50 (a1, b1, c1, d1 & e1) and IC_{90} (a2, b2, c2, d2 & e2) *based isobologram curves (broken line)* for the combinations of CQ with each of the mentioned phytochemicals respectively. The red line that connects the X and Y axes.

Table 2. Antioxidant activity of five tested coumarins, namely; umbeliferon, scopoletin, esculetin, herniarin and 3 aminocoumarin

Compound	Hydrogen peroxide Reducing IC_{50}	Reducing power	DPPH scavenging assay
Umbeliferon	411 µg/ml	133 µg/ml	102 µg/ml
Scopoletin	366 µg/ml	106 µg/ml	125 µg/ml
Esculetin	122 µg/ml	102 µg/ml	83 µg/ml
Herniarin	N.A	N.A	N.A
3-aminocoumarin	733 µg/ml	472 µg/ml	322 µg/ml
Butylated hydroxyl toluene BHT		43 µg/ml	43 µg/ml
Vitamin C	450 µg/ml	89 µg/ml	96 µg/ml

Table 3. Physiochemical properties and the predictive bioactivity of the tested coumarins 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 hydrolipophilicity. PSA (polar surface area) is a measure of the surface area where the polarity is high. 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 standard inhibitors of each of GPCR, kinase and protease enzymes and the nuclear receptors as well as to the standard ion channels modulator

Parameter	Coumarin	Umbeliferon	Scopoletin	Esculetin	Herniarin	3-amino-coumarin
Physiochemical properties						
ClogP	1.79	1.29	1.10	0.80	1.82	0.14
PSA	26.30	46.53	55.77	66.76	35.54	52.33
Natoms	11	12	14	13	13	12
MW (g/mole)	148.16	164.16	194.19	180.16	178.19	163.18
nON	2	3	4	4	3	3
nOHNH	0	1	1	2	0	2
nviolations	0	0	0	0	0	0
nrotb	0	0	1	0	1	0
volume	134.77	142.79	168.34	150.81	160.32	146.09
Predictive bioactivity						
GPCR ligand	-0.90	-0.68	-0.56	-0.58	-0.72	-0.69
Ion channel modulator	-0.48	-0.30	-0.30	-0.25	-0.45	0.07
Kinase inhibitor	-1.25	-0.97	-0.75	-0.85	-0.97	-1.00
Nuclear receptor ligand	-0.75	-0.32	-0.34	-0.30	-0.52	-0.83
Protease inhibitor	-1.13	-0.97	-0.87	-0.87	-0.96	-0.49
Enzyme inhibitor	-0.47	-0.25	-0.19	-0.18	-0.37	-0.21

All the tested coumarins produced a comparable growth inhibitory effect against both *Plasmodium falciparum* K1 and 3D7 indicating that unlike CQ, they act on the same targets in both strains and in a comparable intensity. Nevertheless, their action is still considered weak as compared to CQ as their IC_{50} values against plasmodium growth was within the micro-molar while that of CQ was in the nano-molar range. Furthermore, their

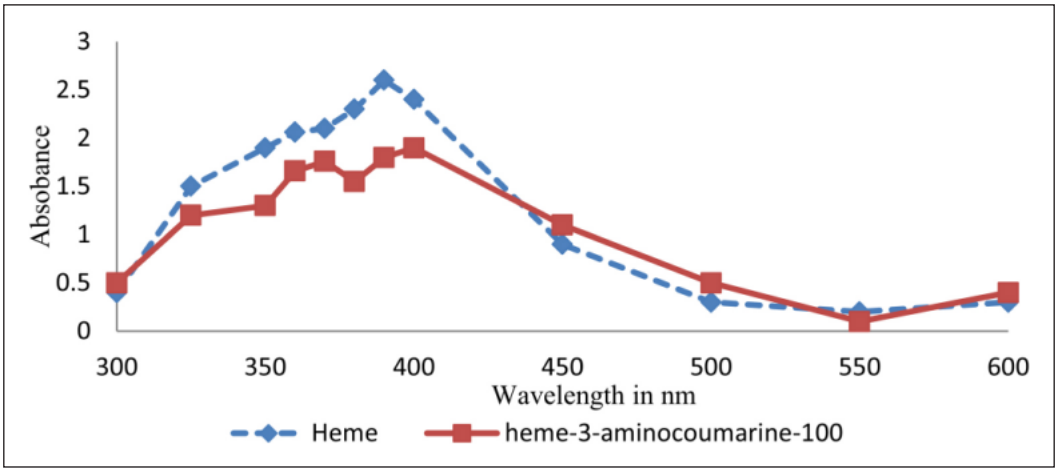

Figure 4. UV-visible spectrum in the range of 300-600 nm of 100 µM of heme solution or a mixture of 100 µM of both heme and 3-aminocoumarin. Soret band was obtained at 390 nm. Lower concentrations of 3-aminocoumarin did not produce any effect on Soret band intensity.

selectivity to plasmodium was high as all of them failed to produce a prominent obnoxious effect on RBCs and Vero cells.

The antioxidant power was seen only in the coumarins that possess free phenolic groups (umbeliferon, esculetin and scopoletin). Ubiquity of phenolic groups confers for the potential of compounds to donate electrons and reduce free radicals. Furthermore, phenolic groups can complex with free ferric ions resulting in fading the latters pro-oxidant activity. Antioxidants act as double edged sword weapons for the cells. From one side they halt the outflow of deleterious free radicals; which are released as byproducts of the cellular activity. On the other hand, they may turn into pro-oxidants and release free radicals at higher concentrations. 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 (Belsare D.P 2010) & (Librado A. Santiago 2014). Previous studies had pointed out to the significance of such discrepancy in eradicating the undeveloped cells (Librado A. Santiago 2014), (Nemeikaitë-Èënienëa 2005) & (Maurya 2010). It is noteworthy that the antioxidant power of the mentioned compounds is not related to their effect on plasmodium as the antioxidant potential of the most powerful one; 3-amino-coumarin, was weaker than the others.

All the tested coumarins failed to affect the potential of merozoites to invade new RBCs. Merozoites invasion is a sophisticated mechanism requires attachment of the merozoites to RBCs membrane and its subsequent internalization. During merozoites invasion, some external merozoite surface antigens, viz; *pf*msp-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 (Pinder J 2002) & (Margos G 2004). This coincides with the notion that as hydrophilic compounds, they don't have any potential to accumulate in RBCs membrane and they don't have the potential to bind to the portals through which merozoites invade the RBCs. (Pinder J 2002), (Margos G 2004) and (Casey 2014).

Ubiquity of phenolic groups in the chemical structure of the compounds suggests that they have a potential to bind to metal center of heme (Pagola, Stephens *et al.*, 2000). In spite of that, all of the tested coumarins including those that possess the phenolic group failed to affect hemozoin formation or slake down Sorret band intensity.

The relatively higher antiplasmodium potential of 3-amniocoumarin absolutely excludes this notion as it is devoid of any phenolic group and does not have any potential to inhibit hemozoin formation. This raises a suggestion of presence of other mechanisms through which such coumarins produce their action.

Antagonism was absent between any of them with CQ. Antagonism with CQ may occur in the presence of any factor that hinders access of CQ to the digestive vacuoles (DV) or inhibits the CQ induced oxidative stress through mopping out the free radicals from the parasite cytosol. On the other hand, all of them failed to produce any synergy or additive effect with CQ except for 3-esculetin, umbeliferon and 3-minocoumarin (section 2.3). Previous studies had set the potential of some compounds to reduce CQ exodus outside the DV and stifling the DV membrane transporters activity as a mechanism to reverse CQ resistance (Sidhu AB 2002). On the other hands, others had pointed out to the importance of CQ induced apoptotic pathway as a target for CQ resistance reversing agents. CQ induced DV membrane permeabilization through inducing minor damage in the DV membrane resulting in exodus of some hydrolytic enzymes that push the machinery to the apoptosis cascade forward (J-H Ch'ng 2011) & (Estelle S. Zang-Edou 2010). It is noteworthy that the impact of 3-aminocoumain combination with CQ is higher in the FIC_{90} based isobologram as compared to the FIC_{50} based one. This indicated that its effect on CQ tolerance is somehow higher than on drug resistance.

It is noteworthy that different coumarins may inhibit cellular proliferation through suppressing growth, cell signaling and metabolism of cells. It was found that they can induce cell cycle arrest at G1 phase through decreasing phosphorelation of PRB and some cyclines, viz, cyclin E and D. They can reduce E2F gene expression that is necessary for DNA synthesis (Weber US 1998). These actions were found on human cells but their ubiquity in plasmodium is not yet confirmed.

Previous studies pointed out to the anti-proliferative role of 3-aminocoumarins and attributed it to the potential of the drug to inhibit the ATPas activity of DNA gyrase enzyme that is responsible for DNA supercoiling. This action propagates cellular death and pushes the machinery of cellular programmed death (apoptosis) forward. Nevertheless this action was seen only on bacterial cells and further studies are required to prove its ubiquity in plasmodium (Wiebke Schröder 2014). Other study found that when it complexes with free metals, 3-aminocoumarin produce a pronounced anti-proliferative activity against bacterial cells (Abdul Amir H. Kadhum 2011). This fact shows some correspondence with results of heme binding and hemozoin formation assays in which 3 –aminocoumarin showed weak potential to slake down Soret band intensity and inhibit hemozoin formation. The metal complexing property of 3 aminocoumarin; as indicated in Abdul Ammer *et al.*, 2011 study (Abdul Amir H. Kadhum 2011), may have conferred for the ability to bind to heme. Compounds bind to heme through establishing π-π interaction with the central iron of the heme moiety (Gorka 2013).

Overall, all the tested coumarins are not ideal to be listed as a conventional anti-malarial therapy due to their relatively weak action as compared with CQ. Furthermore, all of them failed to hinder both CQ resistance and tolerance in *Plasmodium falciparum* K1 *in vitro* except for 3 aminocoumarin suggesting introducing the latter as an adjuvant to potentiate CQ action. The potential of the latter on CQ induced apoptotic pathway is the most plausible mechanism through which it could have synergized CQ. Nevertheless, further studies are recommended to confirm this notion.

Conflict of interest

All authors declare that there is no any conflict of interest regarding publication of this article

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