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ORIGINAL ARTICLE

In vitro Antiplasmodium and Chloroquine Resistance Reversal Effects of Mangostin

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ABSTRACT

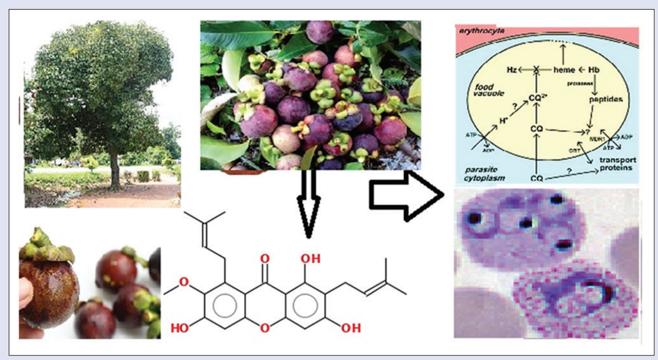
Aim/Background: Chloroquine (CQ) resistance that appeared among different strains of *Plasmodium falciparum* is considered as the worst catastrophe in the realm of malaria chemotherapy. CQ is still the most favorable drug among other antimalarials especially in the poor endemic areas due to its high potency and cost-effectiveness. This urged the scientists to explore for other alternatives or sensitizers for CQ. **Materials and Methods:** In this experiment, the antiplasmodium and the CQ resistance reversing effects of mangostin were tested using the *in vitro* SYBRE green-1-based drug sensitivity assay and the isobologram technique, respectively. Furthermore, its safety level toward two types of mammalian cells, namely Vero cells and red blood cells (RBCs), was screened using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based drug sensitivity and the RBCs hemolysis assays, respectively. On the other hand, its effect against hemozoin formation was screened using β-hematin formation. Meanwhile, its molecular characters were determined the *in silico* on-line free chemi-informatic Molinspiration software for the molecular characterization as well as the standard testes for the measurement of the antioxidant effect. **Results:** Mangostin was moderately effective and selective toward the plasmodium so it is unsuitable to be a substituent for CQ. But it improved the sensitivity of the parasite to CQ. The molecular elucidation suggests that its CQ resistance reversal effect can be ascribed to its ability to interfere with hemozoin formation or the intravacuolar accumulation of CQ. **Conclusion:** Overall, the study suggests mangostin as a possible pharmacophore to develop new CQ resistance reversing agents but further studies are recommended to confirm this notion.

Key words: Chloroquine, falciparum and SYBR green-I, Isobologram, mangostine, resistance

SUMMARY

- Mangostin is an interesting pharmacophore with plenty of pharmacological activities. This study showed that in spite of its prominent activity against the *in vitro* growth of *Plasmodium falciparum*, the idea of its use as a substituent to chloroquine (CQ) is prejudiced as its action is moderate and does not reach to the power of the conventional antimalarials and it had a moderate selectivity toward the plasmodium as it has a detrimental impact on the integrity of the uninfected red blood cells when it was exposed at relatively higher concentrations. However, the study still suggests it in the realm of malaria chemotherapy as it showed a power in reversing the CQ resistance in *P. falciparum* K1 (the CQ resistant strain of *P. falciparum*) especially when they were mixed at ratio as much as 7:3 (CQ/magostin). This action can be ascribed to its impact against hemozoin formation or may be due to its plausible apoptotic effect that might have enhanced the CQ induced apoptosis. Further studies are required for detailed mechanism elucidation and for finding the most optimum combination that produced the best synergy with CQ.

Abbreviations used: CQ: Chloroquine; PBS: Phosphate buffer saline; DMSO: Dimethyl sulphoxide; U.S.A: United States of America; mM: Milli molar; nM: Nano molar; μM: Micro molar; DPPH: 2.2-Diphenyl-1-Picryl-Hydrazyl free radical; Clog P: Octan/water partiction coefficient; PSA: Polar surface area; nON: Number of nonhydrogen atoms; nOHNH: Number of hydrogen donating groups; Nrotb: Number of rotatable bonds; MlogP: Partition coefficient factor (octanol/water partition coefficient); cMCM: Complete Malaria culture medium; min: Minute; PRBCs: Parasitized red blood cells; RBCs: Red blood cells; EDTA: Ethylene diamine tetra-acetic acid; $IC_{50:}$ Inhibitory concentration required to kill 50% of the parasites; IC_{90}: Inhibitory concentration required to kill 90% of the parasites; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATCC: American type culture collection; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SI: Selectivity index; NaOH: Sodium hydroxide; BHT: Butylated hydroxy toluene; NPP: New permeation pathway; *Pfcrt*: *Plasmodium facliparum* chloroquine transporter protein; DV: Digestive vacuole; DVM: Digestive vacuole membrane; $FIC_{50:}$ Fractional Inhibitory concentration for the isobologram analysis for the 50% inhibition of the parasite growth; $FIC_{90:}$ Fractional Inhibitry concentration for the isobologram analysis for the 50% inhibition of the parasite growth



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INTRODUCTION

Malaria is still a major challenging disease in the developing countries due to the resistance toward most of the conventional antimalarial drugs among different strains of *Plasmodium falciparum*.[1] Chloroquine (CQ) is still the most pertinent one due to its relative safety and cost-effectiveness in comparison to the others. But, unfortunately the issue of resistance has compromised its token as an ideal drug.[2,3] This issue urged scientists to search for alternatives or chemo-sensitizers that improves its effect.

Over the recent past, many modern antimalarials have been derived from natural products, viz., artimisnine which was obtained from *Artimisia annua* and prescribed widely in the area wherein CQ resistance predominates. On the other hand, there have been lots of previous studies embarked on the plausibility of reversing drug resistance using phytochemicals obtained from the medicinal herbs. This potential had been proved by some studies, viz., Kirti Mishra Aditya *et al.* 2011; in which the potential of andrographolide; obtained from *Andrographis paniculata,* to synergize other antimalarials was confirmed.[4] In our study, the *in vitro* potential of one of the xanthon derivatives, called mangostin, to inhibit the plasmodium growth and reverse CQ resistance in *P. falciparum* K1.

Furthermore, the molecular elucidation of mangostine effect was studied through screening of its impact on some parasitic molecular targets, viz., hemozoin formation or the new permeation pathways (NPPs) was studied.

Hemozoin represents the innocuous waste products of the heme catabolism. Plasmodium relies solely in hemoglobin as the main source of amino acids and release heme which is detoxified in the plasmodium to hemozoin. Any interference with hemozoin formation results in the accumulation of the toxic heme and the induction of the cascade sequential reaction of the heme induced oxidative stress.[5,6] On the other hand, the NPPs are candidate targets of antiplasmodium drugs. They are expressed on the surface of the plasmodium infected red blood cells (RBCs) as a part of the infection induced cellular remodeling mechanism. They act as portals for nutrients that are difficult to pass through the uninfected RBCs membrane.[7,8]

Xanthones are tricyclic organic compounds; made up of two aromatic rings connected via pyran ring [Figure 1]. They are present in families of Bonetacea and Clausacea and obtained mainly from rinds of mangosteen fruit (*Glacenia mangostina*) and timbers of *Mesua thwaitesii*.[9] The aromatic rings of mangostin is substituted with 3 hydroxyl groups at positions 3, 6, and 8, one methoxy group at position 2 and two 3-methylbutienyl moieties at positions 1 and 7 [Figure 1].[10] Mangostin is an interesting pharmacophore with diverse pharmacological activities including antimicrobial, tuberculostatic, schistosomicidal, antioxidant, astringents, antidiabetic, Anti-diarrheal, gastroprotective, cardiotonic, hepatoprotective, choleretic, and antiprostatic actions.[11-14]

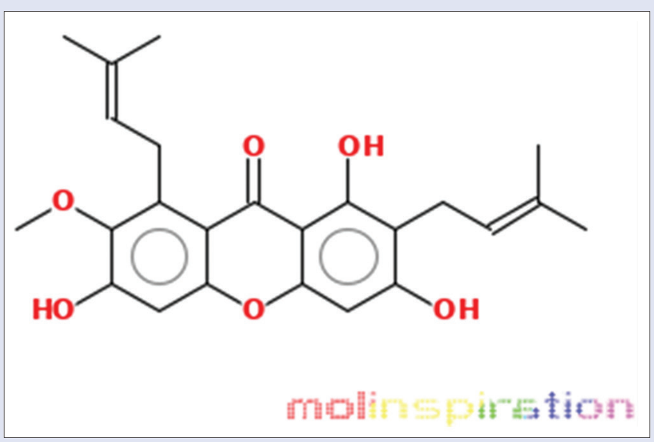

Figure 1: Chemical structure of mangostin as drawn by the Molinspiration simulation software

MATERIALS AND METHODS

Materials and chemicals

RPMI-1640 medium and albumax II, were obtained from Gibco BRL (Grand Island, NY, USA). Meanwhile, each of ethylene diamine tetra-acetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), triton X-100, saponin, hypoxanthine, sorbitol, bovine serum albumin (BSA), (×100) phosphate buffered, dimethysulphoxide (DMSO), phosphate buffer saline (PBS), and CQ diphosphate were purchased form Sigma-Aldrich (St. Louis, MO, USA). Gentamicin was procured from (Jiangxi Dongxu Chemical Technology Co., Ltd), Mangostin was obtained from Indofine Biochemical Company Inc. (Cat No.: A-005). meanwhile, hemin chloride was procured from (IKNOW) (IKONW, certificate number:-GMP, SGS, HALA, KOSHER).

On the other hand, an inoculum of (*P. falciparum* K1) was procured from the institute of Medical Research, Kuala Lumpur Malaysia. Human O + blood was withdrawn taken from the main author under the supervision of an specialized heamatologist. Then it was pelleted and washed using a washing medium containing (25 mM HEPES buffer [pH 7.4], RPMI-1640, 11 mM glucose, 24 mM sodium bicarbonate, and 50 μg/L gentamicin).

Molecular characters assessment

Antioxidant activity measurement

Hydrogen peroxide scavenging activity, reducing power assay, and DPPH scavenging activity were measured as prescribed previously.[15-18] using mangostin at a concentration range of 1 nM-250 μM.

Physiochemical properties calculation and bioactivity prediction

The on-line free chemi-informatic Molinspiration software (http://www.molinspiration.com) was used to determine both of the physiochemical properties and to simulate the bioactivity of mangostin. The software performs a fragment-based virtual screening of different physiochemical properties like; molecular polar surface area (PSA), logarithm of octanol/water partition coefficient (cLOGP), (number of hydrogen donating bonds, number of rotatable bonds, and number of nonhydrogen atoms. Furthermore, the software provides predictive drug-likeness scores toward some intracellular targets, viz., nuclear factors, GPCR, kinase, protease enzyme, and ion channels.

The drug is considered ideal if it does not violate any of the following Lipiniski's rules, viz., moderate lipophilicity (MlogP should be <5), molecular mass <500 Dalton, having no more than 10 acceptor and 5 H donor groups and its (PSA) should be <140 °A.

Parasite culturing, maintenance, and synchronization

Parasites culturing and maintenance

An inoculum of *P. falciparum* K1 was suspended in a culture containing O + RBC suspended in a complete malaria culture medium (cMCM).

The cMCM was prepared by adding 0.5% albumax along with 0.75 mM hypoxanthine to a mixture of the same content of the washing medium. The pH was maintained at 7.4 and the hematocrit was maintained at 2%. The cultured parasites were incubated at the standard conditions (temp = 37°C and an atmosphere containing 5% of each of

CO_2 and O_2 and 90% N_2. Throughout the incubation, the culture medium was changed and the parasitemia progression was checked daily.[19,20]

Parasite synchronization

Synchronization of the the cultured parasites was performed as previously described by Lambros and Venderberge 1979. It is a prerequisites step before running the drug sensitivity assay. Briefly, the process involves incubation of the pelleted unsynchronized parasitized RBCs (PRBCs) with an equal volume of a solution containing 5% (w/v) sorbitol for 10 min and washing out of the sorbitol using the same abovementioned washing medium. The process was repeated thrice.

Synchronization is done so as to lyse the trophozoites or schizonts infested PRBCs leaving the ring infected cells intact. That is why, the process is done while the cultures are predominated with the rings.[21]

Stock and working solution preparation

A stock solution of 100 mM of CQ was prepared using PBS (pH 7.4). It was diluted later to prepare different concentrations of working solutions (1 nM to 1 mM).

On the other hand, the mangostin stock was prepared at 250 µM using DMSO as a cosolvent and the working solutions were in the range of 1 nM to 250 µM. It is worth to note that this concentration was used as the maximum concentration in most of the experiments due to issues related to its poor solubility in water.

Drug sensitivity assay

The drug sensitivity assay to find the antiplasmodium effect of the selected drugs was performed as mentioned earlier by Matthias *et al*. 2010,[22] and Ibraheem *et al*. 2015.[23] Briefly, drug containing 96 well plates; featured triplicates of two folds serial dilution of each 50 µl of each drug (CQ (1 nM to 1 mM) and mangostin (1 nM– 250 µM)), were incubated for 48 hr at 37°C with 50 µl of a PRBCs suspension with a parasitemia and Hct of 2%. (Such that the final HCt and parasitemia would be 1%). Furthermore, control wells were allocated; viz., drug control that is featuring different concentrations of the drug without RBCs, RBCs control which contained the untreated RBCs (0% parasite growth) and the PRBCs control which contained untreated PRBCs at 100% parasite growth. The drugs dilutions were done using working solutions containing the drugs at 1 µM using cMCM as a solvent. After incubation, the plates were freeze-thawed for a while and then 100 µL of SYBR green-I lysis buffer was loaded to each well. The lysis buffer content was (5 mM EDTA, 0.008% saponin, 20 mM Tris, and 0.008% triton-X-100) Then the plate was incubated in dark for 1 hr and at the end, the fluorescence was measured two times after a short plate agitation using Victor Plate reader (Perkin Elmer, Salem, MA) at an excitation/emission wavelength of 485/535 nm. The mean of the two passes was used.[27-29] The experiment was done in triplicates and the results were expressed as mean ± S. E. M of the three trials.

Growth parameters determination

Both of the inhibitory concentrations to kill 50 and 90% of the parasites, respectively (IC_{50} and IC_{90}) for each of mangostin and CQ against *P. falciparum* K1 and 3 D7 were determined after drawing of the log (dose)-response curve using GraphPad prism version 5.

Cytotoxicity against mammalian cells

The cytotoxic effect of mangostin was screened against two models of mammalian cells, namely, the RBCs; wherein the parasite thrives and the Vero cells (renal epithelial cells obtained from monkeys). This step was done to study the selectivity of the drug toward the parasites in comparison to the mentioned cells.

Effect on red blood cells stability

The impact of mangostin against RBCs stability was performed through incubation of Different concentrations of mangostin (1 nM-250 µM) with an RBCs suspension in which the O+ve human RBCs were suspended in an Incomplete Culture Medium iCM containing 25 mM HEPES, RPMI-1640, and 20 µg/ml gentamicin) at 37°C for 48 hr using a 24 well plate (1 ml/cell). Then, 500 µl was withdrawn from each well after a thorough mixing and loaded into Eppendorf tubes. The tubes were centrifuged at 500 g for 5 min and then 200 µl of the supernatants were loaded into a flat bottomed 96 well plate. The absorbance was measured at 540 nm using (Versa Max™) spectrophotometer in order to measure the amount of the released hemoglobin. The results were compared with both negative and positive controls. The former contained RBCs incubated with a drug-free-media. The positive control was produced using 1% of Tween 20 with the RBCs suspension so as to produce complete RBCs hemolysis. The RBCs hemolysis at each concentration was calculated as follows.

$$\%\text{ of hemolysis} = \frac{As - An)}{Ap - An)} \times 100 \rightarrow$$

Whereas As An and Ap are absorbance values of the test sample, and both negative and positive controls respectively.

Effect on Vero cells

The cytotoxic impact of mangostin against Vero cells (American type culture collection) was done through 48 h incubation of mangostin (1 nM-250 µM) at the mentioned standard incubation conditions and in a culture medium containing 10% BSA, RPMI-1640 and the antibiotic mixture (100 U/ml penicillin and 100 µg/ml streptomycin).

After incubation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed.[24] Then the dose-response-curve was extrapolated and the IC_{50} of mangostin against vero cells was determined using GraphPad Prism version 5.

Selectivity index

The cytotoxicity against the pathogen was compared to that against Vero cells and the RBCs through calculation of the selectivity index (SI) (ratio of the drug IC_{50} against one of the model cells of mammals, such as; RBCs and Vero cells to that against plasmodium). SI points out to the ability of the compound to inhibit the parasite growth without affecting the mammalian cells. The drugs are considered non selective if their SI is below than 10 and moderately selective if it was between 10 and 100.

Drug combination assay and isobologram analysis

To study the impact of mangostin and CQ combination on the plasmodium growth, the isobologram technique that was described previously by Zaid *et al*. 2015 was adopted.[23,25] Briefly, working solutions of each of CQ and mangostin were prepared from their stocks at 16 times their IC_{50}. This dilution was chosen in order to have the IC_{50} of each drug falls in the fourth twofold serial dilution. After that, the two solutions were mixed at (10:0, 7:3, 5:5, 3:7, and 0:10; ratios of CQ/phytochemical). Then, 50 µl of each of the mentioned combination was loaded in triplicate in row H of the l plate (H2-H11) and were serially diluted within the plate (rows G-B). The peripheral wells were loaded with 50 µl of the controls (drug, PRBCs, RBCs controls). Then the mixture was incubated at the mentioned standard incubation conditions for 48 hr and was treated as in the drug sensitivity assay. The parasite growth profile was determined to estimate each of the IC_{50} and IC_{90} of each combination separately.

For each combination, both of the fractional inhibitory concentration (FIC_{50} and FIC_{90}) were calculated through finding 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.

$$FIC_{50} = \frac{IC_{50}\text{ of the drug in combination}}{IC_{50}\text{ of the drug when it is incubated alone}} \quad FIC_{50} = \frac{IC_{90}\text{ of the drug in combination}}{IC_{90}\text{ of the drug when it is incubated alone}}$$

$$\text{Total } FIC_{50} = FIC_{50}\text{ CQ} + FIC_{50}\text{ andro} \quad \text{Total } FIC_{90} = FIC_{90}\text{ CQ} + FIC_{90}\text{ andro}$$

At the end, both of the FIC_{50}- and FIC_{90}-based isobolograms were plotted. The values of FIC_{50} values for each of mangostin and CQ were extrapolated on the abscissa (X-axis) and ordinate (Y-axis), respectively, for each of the FIC_{50}- and FIC_{90}-based isobolograms. The line that links the two drugs FICs (while their FIC = 1) is theline of additivity. The interaction is deemed as additive if the points fell on that line or the total FIC is equal to 1. It is deemed as synergistic when the points fell below the line. Furthermore, it is considered as antagonistic if the points occur above the line, respectively, or the total FIC is >2.[23,25]

The molecular elucidation study

Effect on sorbitol induced hemolysis of parasitized red blood cells

The effect on parasite induced permeability pathway was investigated using the well-known sorbitol induced hemolysis as previously described.[26,27]

β-hematin formation assay

β-hematin formation assay is based on the drug interaction with hemin chloride and its ability to interfere with the hemozoin polymerization. Briefly, 100 µl of each test compound (0.8–40 mM) was added to an equal volume of 8 mM hemin chloride (dissolved in DMSO), in Eppendorf tube (i.e., the ratio of the drug to hemin will be 0.1–5 molar equivalents (drug/hemin chloride).

Furthermore, control tubes containing D. W instead of the drug were allocated. Then, β-hematin formation was induced through adding 200 µl of 8 M acetate buffer (pH = 5). After that, the tubes were left at 37°C for 18 hr and centrifuged at 3000 g to pellet out the β-hematin. Later on, the pellet was dissoluted in DMSO and re-centrifuged to remove the unreacted hematin. The latter suspends in the supernatant, leaving a second pellet that contains the the pure β-hematin.

After that, 400 µl of 0.1 N NaOH was loaded to each tube so as to dissolve β-hematin. Then 100 µl aliquots of the final solution were transferred to other tubes, diluted 4 times using the mentioned NaOH solution and the absorbance was measured spectrophotometrically at at 390 nm. Finally, the absorbance versus concentration curve of mangostin was compared with that of CQ. CQ is a model drug for interference with β-hematin formation.[28]

RESULTS

Antioxidant activity of mangostin

Mangostin revealed an antioxidant activity. Its activity is still deemed to be quietly less than that of reference comparator antioxidants, viz., Vitamin C or butylated hydroxyl toluene. It showed a stronger free radical scavenging activity against DPPH a lipid-soluble free radical [Table 1].

In silico molecular characterization

The molinspiration software suggests that mangostin is a highly lipophilic compound with clog *P* value >6. This disqualifies it as an ideal candidate for drugs with acceptable pharmacokinetic properties but it is still can be studied *in vitro* as a pharmacophore for development of other drugs with a stronger therapeutic effect and less undesirable characters. The software revealed a noticeable effect for mangostin against enzymes and nuclear receptors [Table 2].

Drug sensitivity assay (effect against plasmodium, red blood cells, and Vero cells)

As per (Li *et al.* 2009),[25] according to the IC_{50} values, which were determined using the SYBER GREEN-1-based hypersensitivity assay, the compounds are categorized into groups of different potency level [Table 3].

Unlike CQ whose potency was excellent (IC_{50} <1 µM) as per the (Li *et al.* 2007) criteria, mangostin showed a good potency against *P. falciparum* 3D7 and K1 [Table 3a and b]. Furthermore, its effect was comparable against the two falciparum strains. It is about 470 and 35 times less potent than CQ against the mentioned strains, respectively. This discrepancy disqualifies it to be a substituent of CQ [Table 3b].

The compound is deemed as cytotoxic against mammalian cells only if its IC_{50} >30 µg/ml. Table 3b shows that the cytotoxic effect of each of mangostin and CQ was higher than the molar equivalents of the cytotoxicity threshold (30 µg/ml) so both of them are not considered as non-toxic. But mangostine was relatively more toxic than CQ as it showed low toxicity to the mentioned cells while CQ did not show any effect [Table 3].

Mangostin was ostensibly less selective to the plasmodium cells when its selectivity index was compared with that of CQ. It showed a moderate selectivity and this is considered as another drawback for its use as an antimalarial drug [Table 3b].

Isobologram analysis

Synergy was observed when mangostin was mixed with CQ especially when the two were mixed at a ratio of 7:3 (CQ/mangostine). It was mild to moderate as the total FIC_{50} and the FIC_{90} values were in the range of 0.5–1 and the points in the curve were slightly or moderately below the additivity line [Figure 2 and Table 4]. The interaction was more obvious in the isobologram drawn based in the IC_{90} indicating that mangostin was more powerful in prevention of the CQ tolerance at different mixing ratios. At the higher mangostin/CQ ratio, the interaction approached the additive effect especially in the FIC_{50}-based isobologram as the pointes were approaching the line of the additivity [Figure 2].

Table 1: Antioxidant effect of mangostin using 2.2-diphenyl-1-picryl-hydrazyl free radical, hydrogen peroxide scavenging activity or reducing power assay

Compound	Hydrogen peroxide reducing IC_{50}	Reducing power	DPPH scavenging assay
Mangostin	53 µM	77 µM	33 µM
Butylated hydroxyl toluene BHT		13.1 µM	24.4 µM
Vitamin C	20.1 µM	41.2 µM	

BHT: Butylated hydroxyl toluene; DPPH: 2.2-Diphenyl-1-Picryl-Hydrazyl free radical

Table 2: The *in silico* molecular characters of mangostin

Physiochemical character	Value	Cellular target	Molinspiration bioactivity score
ClOGP	6.32	GPCR ligand	0.03
TPSA	100.13	Ion channel modulator	−0.06
Natoms	30	Kinase inhibitor	−0.05
MW	410	Nuclear receptor ligand	0.49
nnOHNHON	6	Protease inhibitor	−0.15
Nviolations	3	Enzyme inhibitor	0.45
Nrotb	1		

Nrotb: Number of rotatable bonds

Table 3: Cytotoxicity of mangostin and chloroquine against *Plasmodium falciparum*, red blood cells, and Vero cells. It is subdivided into Tables 3a and b

a. The potency classification of compounds against *P. falciparum*	
Drug IC_{50} range	**Extent of the potency**
<1 µM	Excellent potency
1µM-20 µM	Good activity
20 µM-100 µM	Moderate activity
100-200 µM	Low activity
>200 µM	Inactive

b. The Cytotoxic effect of mangostin against *P. falciparum* (3D7 and K1), Vero cells, and red blood cells		
Part A	**Mangostin**	**Chloroquine**
Gram/weight (g/mole)	396	515
Molar conc. (µM) equivalent to 30 µg/ml	75.5	58
Part B (IC50 values against P. falciparum 3D7 and K1, Vero cells, and RBCs		
IC_{50} against RBCs in µM	178.6±6.8	>1000
IC_{50} against Vero cells in µM	200.6±11.3	>1000
P. falciparum 3D7 µM	9.4±1.03	0.021±0.002
SI compared to RBCs	18.9	Very high
SI compared to Vero cells	21.3	Very high
P. falciparum K1 µM	9.7±0.93	0.265±0.05
SI compared to RBCs	18.3	Very high
SI compared to Vero cells	20.6	Very high
Part C (IC90 values against P. falciparum 3D7 and K1, Vero cells, and RBCs		
IC_{50} against RBCs in µM	634.6±19.3	>1000
IC_{50} against Vero cells in µM	>1000	>1000
P. falciparum 3D7 µM	45.6±2.8	0.043±0.001
SI compared to RBCs	14	Very high
SI compared to Vero cells	High	Very high
P. falciparum K1 µM	47.3±2.9	0.92±0.05
SI compared to RBCs	13.5	Very high
SI compared to Vero cells	High	Very high

The table provides in subpart A as the gram/weight of each item and the concentration limit in µM that is equivalent to 30 µg/ml, in subpart B, The IC_{50} values against the mentioned calls as well as the IC_{50}-based selectivity indices. And in subpart C of Table 3b, the IC_{90} values against the mentioned cells as well as the IC_{90}-based selectivity indices are listed. *P. falciparum: Plasmodium falciparum*; SI: Selectivity Index; RBCs: Red blood cells

Molecular elucidation

Heme polymerization and β-hematin formation was inhibited by mangostin (IC_{50} = 4.2 mM) which was about 80 times less than that of CQ (IC_{50} = 53 µM). Meanwhile, mangostin failed to produce any effect on the NPPs.

DISCUSSION

The possible use of natural products against malaria has been studied extensively. Only few of the natural products proved efficiency in the clinical field, such as; *Artimisia annua*. The research is going on to explore for more products with an antiplasmodium or drug resistance reversal potentials.[30] Our study aimed at finding the possible use of mangostine; one of the famous phytochemicals, as an antiplasmodium or CQ resistance reversing agent.

Mangostin is a xanthone derivative; made up of two aromatic rings connected via pyran ring. Its chemical structures [Figure 1] suggests that it is highly lipophilic as it has an lipophilic core; substituted with two (-3-methylbutienyl) moieties at positions 1 and 7. Although it has some hydrophilic groups, viz., three hydroxyl groups at positions 3, 6, and 8 and one methoxy group at position 2. Its lipophilicity was confirmed by the results of the hydrolipophilicity indices of the Molinspiration simulation software [Table 2]. This suggests that it can accumulate in the double layered plasma membranes and disrupt their functions, an this was obviously seen in its obnoxious impact on the RBCs stability [Table 3]. This character compromises its selectivity toward the plasmodium as compared to RBCs and this impact should be taken into consideration whilst suggesting it to curb the plasmodium growth. Accumulation of lipophilic compounds in RBCs membrane induces some structural changes characterized by disruption of the double layered membrane integrity resulting in membrane speculation, alteration in RBCs morphology and subsequent RBCs hemolysis.[31] Paradoxically, the *in vitro* assessment of its antioxidant potential suggests an antioxidant and free radicals scavenging effects for mangostin as seen in the results of the DPPH assay. This may confer some protection to the membrane but its protection may incur during its early accumulation within the membrane but hemolysis is induced when its accumulation exceeds the thresholds.

Results of the *in silico* Molinspiration software (http://www.molinspiration.com) showed that mangostin violated one of the Lipiniski rules criteria that it showed a lipophilicity higher than the upper threshold. This disqualified it as a candidate drug, but in spite of that, our study is still suggesting it as a pharmacophore to develop other derivatives that hold its pharmacodynamic properties but have different pharmacokinetic ones or to formulate it in a dosage form that ensures its delivery into the site of action. For instance, formulation of the drug in a form of nano particles, liposome or using any of the technologies related to the nano technology. It is worth to note that in spite of its inappropriateness to be candidate as a pharmaceutical drug as per the

Table 4: Results of the fractional inhibitory concentration$_{50}$ and fractional inhibitory concentration$_{90}$-based isobolograms for chloroquine/andrographolide mixtures

Mixing ratio (CQ/mangostin)	IC_{50} CQ	IC_{50} mangostin	FIC_{50} CQ	FIC_{50} mangostin	Total	Mixing ratio (CQ/mangostin)	IC_{90} CQ	IC_{90} mangostin	FIC_{90} CQ	FIC_{90} mangostin	Total
10/0	265.50±5.25	0.00±0.00	1.00	0.00	1.00	10/0	756.07±14.94	0.00±0.00	1.00	0.00	1.00
7/3	127.11±2.41	1906.61±44.30	0.50	0.22	0.72	7/3	349.67±50.54	6259.95±871.42	0.55	0.14	0.69
5/5	128.34±2.54	4489.49±38.20	0.52	0.50	1.02	5/5	300.66±5.94	19593.62±2982.66	0.47	0.44	0.91
3/7	96.00±7.20	7832.45±50.20	0.35	0.60	0.95	3/7	210.14±40.18	22365.44±298.60	0.32	0.50	0.82
0/10	0.00±0.00	9078.79±59.00	0.00	1.00	1.00	0/10	0.00±0.00	44503.68±124.60	0.00	1.00	0.00

CQ: Chloroquine; FIC: Fractional inhibitory concentration

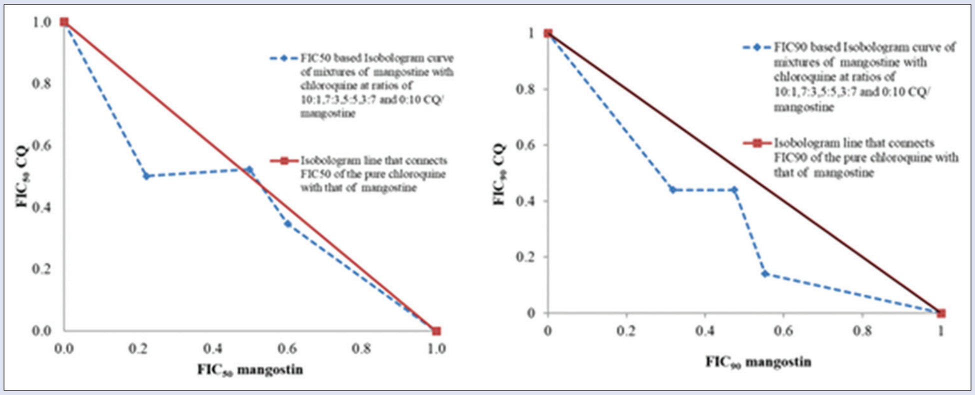

Figure 2: The FIC_{50}- and FIC_{90}-based isobolograms for different combination mixtures of mangostin and chloroquine at (10:0, 7:3, 5:5, 3:7 and 0:10 (chloroquine/ mangostin). The red lines in the two graphs represent lines of additively. Synergy is considered for the points located above the line of additivity while antagonism is considered for points located above that line

results of the simulation software, mangostin has been included in lots of *in vitro* study and its action against cellular growth and the molecular machinery of different cells was studied extensively.[12,14,32]

The study revealed a poor effect for mangostin against all the targets except on the nuclear receptors as an enzyme inhibitor. Nuclear receptors are targets of the transcription factors which promote or repress different genes expression. This effect against different cell line was proved previously as its effect on the expression of the inflammatory genes[32] or its effect on genes involved in the differentiation of myeloblasts to myotubules.[33] On the other hand, the software predicted the presence of an inhibitory effect against intracellular enzymes. This action for mangostin was seen against different intracellular enymes, such as; cyclooxygenase enzyme that is involved in inflammation in mammals,[13] sphingomylinase that is involved in apoptosis or on enzymes involved in cellular mitosis like topoisomerase or DNA polymerase.[34] But any way such effects were detected *in vitro* in human and were suggested by the software in the human models. But this suggests their presence in plasmodium as well due to the homology in different intracellular targets with the human model. The selective effect against the parasite can be attributed to having a larger extent of action against the parasite rather than the mammal cells.

The *in vitro* assessment of hemozoin formation inhibition shows that mangostin had a capacity to bind to heme and suppress hemozoin formation in an extent lower than that of CQ. This can be attributed to its quinone group containing structure that qualifies it to establish bindings with heme.[35,38] Ubiquity of the lipophilic alkyl side chains in the mangostin structure might have hindered its binding to heme [Figure 1]. Heme is a toxic byproduct of hemoglobin catabolism. It is detoxified inside the digestive vacuole (DV) through series of biocrystallization and biomineralization steps to produce hemozoin as an innocuous waste product. This step is crucial for the survival of the parasite and may be targeted by many drugs that lead it its accumulation within the plasmodial cytosol. It has a powerful pro-oxidant effect and cellular damage. Interference with heme detoxification is the main mechanism of CQ; the most widely used conventional antimalarial chemotherapy.[36] Hemozoin formation requires establishment of reciprocate iron oxygen bonds between the central iron of the one of the ferroprotoporphyrine moieties and the carboxyl group of the other (π-π bonding).[37] This bonding results in creation of heme dimers that can stack together though establishing hydrogen bonds among the uncoordinated side chains. This process can be inhibited by drugs that can establish π-π bond with the feroprotoporphyrine resulting in halting of heme dimer and the subsequent hemozoin formation. CQ is a good example as it contains hydroxyl moieties that entitle it to undergo this π-π bonding.[37] its ability to reduce Sorret band intensity suggests that it can inhibit heme polymerization. Drugs that interfere with hemozoin formation may reduce its action through establishing the π-π bond, induction of heme aggregation or precipitation or through creation of axial bonds through binding with the feroprotoprphyrine oxygen at an axial position.[38] The similar stoichiometric ratio of mangostin to that of CQ in the heme binding assay, suggests that their binding to the ferroprotoporphyrine occurs through π-π binding.

The inhibitory effect against plasmodial hemozoin formation was not parallel with that against the parasite growth as the former required higher concentration. This discrepancy may be due to factors related to the drugs ability to accumulate inside the DV as each needs to cross the DV membrane and accumulate against the drug efflux mechanisms. Furthermore, this phenomenon suggests that, the antiplasmoial action is not conferred only by their anti hemozoin action but other mechanisms are suggested.

Different antimalarials target different intra-cellular pathways as some act on the DV, viz., the parasite protease enzyme or heme detoxification pathways. Others affect cytoplasmic targets like fatty acid or isoprenoid synthesis pathways, histidin-rich protein, or plasmodial protein kinase.[39] Not only does CQ acts on the hemozoin pathway, recently, it has been found that it may act first as a lysmotropic amine, like CQ. It can bind to the integral proteins of the DV membrane resulting in the permeabilization of its low gram/weight hydrolytic enzymes to the cytosol, viz., cathepsin. The later trigger the sequential cascade of apoptosis induction.[40]

At low doses, CQ induces the apoptotic features at a basal level while at higher doses (micro-molar concentrations), a higher number of apoptotic cells with MOMP and caspase over-activation evolve. Meanwhile, at the physiological nanomolar concentration, CQ accumulates inside the DV and starts interfering with hemozoin formation. It starts appearing in the cytosol when its concentration jumps to micro-molar concentration due to the DV membrane permeabilization.[16,40]

The study excluded any effect for mangostin against the NPP pathway within their effective concentrations against the parasite growth (data not shown). The NPPs evolve due to intraerythrocytic ubiquity of the parasite which induces structural changes in the RBCs membrane characterized by their appearance. NPPs are specific channels that regulate entry of the nutrients and electrolytes and enhance exodus of the waste products within the infected cells only. Its inhibition may compromise the parasite growth.[41,42]

Mangostin showed a prominent antioxidant power. Antioxidants act as double edged sword weapons for the cells. Form one side; they protect the cells through halting the flow of the deleterious free radicals; which are released as by products due to the cellular activities. On the other hand, 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.[43,44] Previous studies had pointed out to the significance of such discrepancy in eradicating the undeveloped cells.[44] Thus, it is recommended to test their impact on free radicals accumulation at the concentration wherein their antiplasmodium impact had been produced.

Unlike most of the phytochemicals, mangostin had a low toxic effect against two models of mammalian cells; RBCs and Vero cells. But it is still considered as a patent drug as it produced its cytotoxicity at IC_{50s} >30 μg/ml [Table 3]. This rendered it moderately selective drug to the plasmodium. Consequently, caution should be exercised while introducing it to malaria chemotherapy. Its impact on the RBCs can be attributed to its lipophilicity which qualifies it to accumulate in the cell membrane and induces structural changes characterized by disruption of the membrane double layer integrity, membrane speculation, alteration in RBCs morphology and subsequent RBCs hemolysis.[30] Paradoxically, the *in vitro* assessment of its antioxidant potential suggests that mangostin confer some protection to cell membranes through scavenging the deleterious free radicals as it showed a prominent potential to scavenge the lipophilic DPPH. This protection may incur during its early accumulation within the membrane but hemolysis is induced when its accumulation exceeds the thresholds.

On the other hand, its effect against Vero cells can be ascribed to its aptitude to induce the apoptotic pathway and cell cycle arrest.[45] Its lipophilicity qualified it to disrupt the functional characters of the membranous organelles like the mitochondria and the lysosomes resulting in the induction of the cascade pathway of the apoptosis. Furthermore, it may disrupt the integrity of the cell membrane resulting in changing the physiological function of the cells. The results revealed a comparable cytotoxic effect against the teste mammalian cells but this does not exclude a discrepancy in the mechanism through which the cytotoxicity was produced in the RBCs and the vero cells.

Different drugs were tested for the CQ -resistance reversal using different CQ resistant strains of *P. falciparum*. Some showed good effect like; calcium channel blockers, antipsychotics, tricyclic antidepressants, antihistamines, and nonsteroidal anti-inflammatory drugs.[46] It is suggested that their action is through inhibition of the functional activity of *pf*crt; the channel protein involved in the accumulation of CQ within the DV.[47] Previously, it was reported that CQ resistance is closely associated with mutational changes in the *pf*crt structure[48] and its function is affected by the biochemical changes within the surrounding environment. For instance, the -reversing-effect of verapamil was ascribed to its binding to certain allosteric sites within the *Pf*crt. But till now, a clear molecular elucidation for its claimed action has not been achieved yet.[49]

All in all, any drug; that may enhance the mentioned mechanisms, may synergize CQ and reverse its resistance. Since phytochemicals are Janus molecules and can act on multiple intracellular targets. They may chemo sensitize CQ and reverse its resistance.

Furthermore, CQ synergism may be conferred by drugs that enhance its binding to the heme moiety or its intra-vacuolar accumulation or those that compromise the DV membrane (DVM) stability and increase its permeabilization. This is followed by seeping of the hydrolytic enzymes into the parasite cytoplasm and induction of the apoptosis. It was suggested that drugs which augment CQ induced apoptotic pathway may confer synergy.[40,50]

Results of the isobologram analysis revealed absence of any antagonism between CQ and mangostin as none of the combinations produced a sum for FIC_{50} tot or FIC_{90} >2. Antagonism with CQ may occur in the presence of any agent that interfere with access of CQ to the DV or inhibits the CQ induced oxidative stress through moping out the free radicals. Although, both phytochemicals have had good antioxidant potential. But this could not have entitled them to antagonize CQ effect.

Synergy was obtained only when mangostin was combined with CQ especially when both were combined at a ratio of 7:3 (CQ/mangostin). At this ratio, mangostin concentration was as little as 3 μM suggesting higher selectivity for its synergy with CQ. Previous studies have attributed the potential of CQ resistance reversing agents to their ability to reduce CQ exodus outside the DV by inhibiting the DV membrane transporters. Others suggested that CQ induced apoptotic pathway can be set as a target for some drugs to sensitize CQ.[40,41] It is noteworthy that its synergy was somehow more obvious in the FIC_{90}-based isobologram rather than the FIC_{50} based one suggesting that its potential to inhibit CQ tolerance is higher than its effect on the resistance.

Mangostin effect against hemozoin formation can be set as another reason for the observed synergy at the mentioned ratio or the additive effect that was obtained at the combinations of the higher mangostin ratio. Previous studies have pointed out to the role mangostin in induction of the apoptotic pathway and induction of cell cycle arrest in human cancer cells. Such action may be conferred by drugs that can bind to nuclear receptors; an action which is suggested for mangostin as per results of Molinspiration simulation software. This suggests that this pathway might have imparted in the induction of the CQ synergy with mangostin, but further studies are required to confirm this notion.

CONCLUSION

Overall, although, it is unsuitable to use mangostin as a substituent for CQ, it can be considered as an important pharmacophore to develop new antimalarials in the future. The inappropriateness stems from its strongly lipophilic properties that interferes. Mangostin has a promising effect against hemozoin formation both *in vivo* and *in vitro* and this paves the way to develop new derivatives that retain this activity. Its synergy with CQ suggests its use as a sensitizer but further structural or pharmaceutical modifications are required to improve this action.

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Conflicts of interest

There are no conflicts of interest.

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