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In vitro investigation into the Haemin Polymerization Inhibitory activity of chloroquine and emetine dihydrochloride hydrate

Abdullahi Muhammad Daskum

daskum341@gmail.com

Department of Biological Sciences, Yobe State University, PMB 1144, Damaturu.

Abstract

During the intra-erythrocytic development of malaria parasites, haemoglobin in the red cell cytosol is ingested and taken to the food vacuole, where it is degraded. Haemoglobin degradation in the malaria parasite results in the formation of heme/haematin, an essentially toxic protein and denatured globin fragments. Due to its toxicity, free heme (Fe^{+3}) induce oxidative stress by generating reactive oxygen species, subvert and lyse membranes, as well as inhibit the action of a number of enzymes, thus leading to parasite death. To overcome the toxicity of free heme (Fe^{+3}) and avoid death, malaria parasites convert free heme (Fe^{+3}) to a non-toxic haemozoin. This research intends to demonstrate the *in vitro* conversion of haemin to β-haematin/haemozoin through haemin based spectrophotometry and test emetine dihydrochloride hydrate and chloroquine diphosphate for their ability to prevent the conversion of haemin to β-haematin. Results obtained justify the inhibitory activity of chloroquine on the haemin polymerization pathway, but the β-haematin inhibitory activity of emetine remains inconclusive. Whilst the study did not confirm the *in vitro* β-haematin inhibitory activity of emetine, more research is required to determine the exact pathway to which emetine act upon in the malaria parasite and more broadly, further investigation into its inhibitory activity on the haemin polymerization pathway is needed.

Keywords: Haemin, β-haematin, Emetine dihydrochloride hydrate, Chloroquine diphosphate, Haemoglobin degradation.

INTRODUCTION

Most of malaria cases in the world are due to one of the four species of *Plasmodium* parasites that largely infect humans; *Plasmodium falciparum, P. vivax, P. ovale,* and *P. malariae*. Amongst these four species of malaria parasites found to cause disease in humans, *P. falciparum* is the most virulent and causes most of the deaths while *P. vivax* is the most predominant (Flannery et al., 2013). Parasites that cause malaria all develop through the same general life cycle, which alternates between the human host and *Anopheles* species of mosquito. In human, exo-erythrocytic and intra-erythrocytic development occurs. During the intra-erythrocytic/blood stage development, haemoglobin in the cytosol is ingested (Baelmans et al., 2000) and taken to the food vacuole, whose pH is estimated at 5.0-5.4, where it is degraded (Coronado et al., 2014).

Haemoglobin, an oxygen-carrying pigment and major component of a mature red blood cell, is normally formed as a tetramer of two-α-globins and two-β-globins chains (Taylor et al., 2013). Approximately 95% of proteins in the red cell cytosol is composed of haemoglobin, reaching a concentration of about 5 mM inside the cytoplasm (Coronado et al., 2014). Haemoglobin degradation in the malaria parasite results in the formation of heme/haematin, an essentially toxic protein (Baelmans et al., 2000) and denatured globin fragments. Aspartic proteases - plasmepsins and cysteine proteases - falcipains have been implicated as the major mediators of this degradative process (Moura et al., 2009).

Plasmepsins I and II initiate haemoglobin degradation by cleaving the peptide bond between phe-33 and leu-34 at the hinge region of α-globin, thereby unravelling and exposing the haemoglobin to further digestion (Lolupiman et al., 2014).

Denatured globin are thought to be hydrolysed further by falcipains (the cysteine protease) and zinc containing metallopeptidase (falcilysin) into peptides and later to smaller peptides respectively (Eggleston et al., 1999), which are expected to be transported to the parasites' cytoplasm by peptide transporter located in the food vacuole (Kumar et al., 2007). While denatured globin are hydrolysed and transported to the cytoplasm, heme accumulates as crystalline particles within the digestive vacuole (Goldberg et al., 1990).

Peptides are hydrolysed into amino acids by exopeptidases in the cytosol and used for protein synthesis by the parasites (Kumar et al., 2007) while the build-up of the crystalline particles in the digestive vacuole may result in the rise of the level of free heme (Fe^{+3}) to about 300-500 mM (Tekwani and Walker, 2005). Due to its toxicity, free heme (Fe^{+3}) induces oxidative stress by generating reactive oxygen species, subvert and lyse membranes, as well as inhibit the action of a number of enzymes, thus leading to parasite death (Kumar et al., 2007). To overcome the toxicity of free heme (Fe^{+3}) and avoid death, malaria parasites develop a means of converting free heme (Fe^{+3}) to a non-toxic haemozoin. The free heme is sequestered into haemozoin/malarial pigment and the degradation of free heme (Fe^{+3}) is facilitated by hydrogen peroxide within the parasite's food vacuole or glutathione-dependent degradation which occurs in the parasite's cytoplasm (Biochemistry of Plasmodium, 2014). In both cases, elemental mapping of transmission electron micrographs by electron energy loss spectroscopy conclude that haemozoin formation is the overwhelming fate of heme released in the parasite (Egan, 2008).

Although many studies have been put forward to discover drugs effective against the malaria parasite, drugs such as antifolates (sulfadoxine-pyrimethamine) target the folate pathway (Nzila, 2006 a), while the haemin polymerization pathway in the food vacuole, the mitochondrion, the translationally controlled tumour protein and Ca^{2+} pump localized in the endoplasmic reticulum (Figure 1) otherwise termed PfATP6 are potential targets for artemisinin antimalarials (Krishna et al., 2008). In addition, previous findings indicate that artemisinin antimalarials have broad stage specificity, from the rings through to trophozoite and shizonts. However, chloroquine and artemisinin combination therapies (ACTs) were thought to target the haemin polymerization pathway.

Quinoline antimalarials remained the gold standard in the treatment of malaria for more than half a century. Despite the wide use of quinolones and the amount of time invested on research, the exact mechanism of action of these frontline antimalarials still remain unclear (Pandey et al., 2001). Though, many theories aimed at describing the exact mechanism of action of quinoline antimalarials have been put forward, it is expected that most of the erythrocytic schizonticidal antimalarials target the haemoglobin degradation pathway of the malaria parasite (Pandey et al., 2001). Chloroquine in particular, was previously reported to be active against the blood stage of malaria parasite's development only, and not stages like hypnozoites, pre-erythrocytic or mature gametocytes (Foley and Tilley, 1998).

Quinolines as weak bases were thought to accumulate the parasite's acidic food vacuole, thereby reaching high concentrations (Pisciotta and Sullivan, 2008). However, chloroquine accumulation can be well defined on the basis of ion-trapping mechanism. Being a diprotic weak base, chloroquine penetrates membranes of infected RBCs, and move down the gradient to accumulate the acidic digestive vacuole in its unprotonated form. Upon reaching the digestive vacuole, the drug becomes deprotonated (Foley and Tilley, 1998). Deprotonation of chloroquine in the low pH compartment prevents it from permeating out through the vacuole's membrane, hence its concentration.

Concentration of this antimalarial compound in the food vacuole was shown to dimerize with ferriprotoporhyrin IX, leading to inhibition of heme to haemozoin polymerization (Figure 2), that eventually result in the accumulation of the toxic heme or heme-chloroquine complex in the parasite's food vacuole. In addition, previous reports show that chloroquine inhibit haemoglobin transport vesicle trafficking, with a resultant accumulation of haemoglobin and vesicles (Roberts et al., 2008). Furthermore, it is expected that parasite feeding is affected by the accumulation of free heme that prevent vital digestive enzymes like vacuole protease to properly function (Bray et al., 2005). The parasite is killed when heme-chloroquine complex accumulate in the parasite membranes, thus destroying the membranes by lysis and lipid peroxidation mechanism (Bray et al., 2005).

This study was aimed to determine the antimalarial activity of emetine dihydrochloride hydrate and chloroquine diphosphate. The research also intends to demonstrate the *in vitro* conversion of haemin to β-haematin through haemin based spectrophotometry and test emetine dihydrochloride hydrate and chloroquine diphosphate for their ability to prevent the conversion of haemin to β-haematin.

MATERIALS AND METHODS

β-Haematin Formation Assay

In order to demonstrate the *in vitro* conversion of haemin to β-haematin/malaria pigment and to measure the inhibitory effect of chloroquine and emetine, the first protocol previously described by Baelmans et al. (2000) with little modification was used.

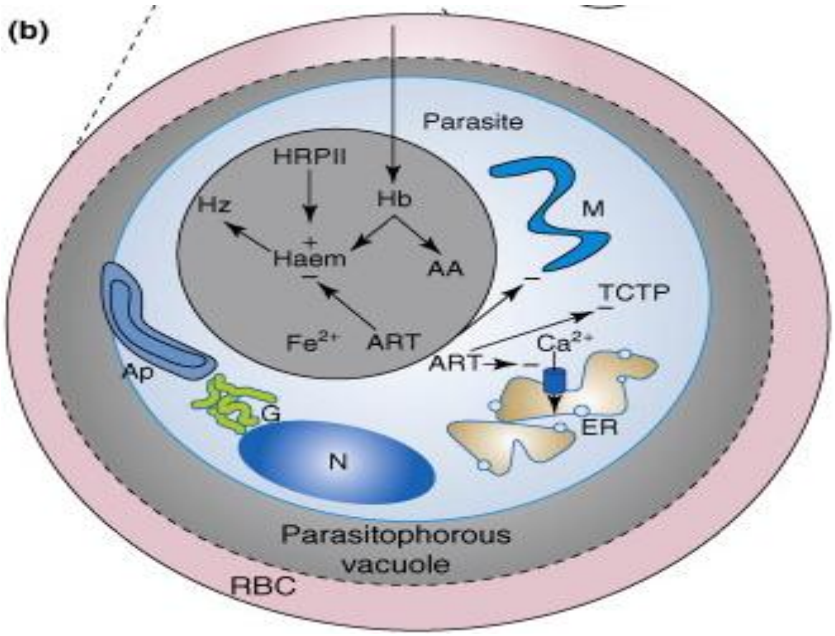

Figure 1. Potential targets of artemisinin antimalarials indicated by arrows with bar: Hb- Haemoglobin, HRP II- Histidine Rich Protein II, Hz- Haemozoin, AA- Amino-acid, ART- artemisinin, M- Mitochondria, TCTP- Translationally controlled tumour protein, ER- Endoplasmic reticulum, N- Nucleus, G- Golgi apparatus, Ap- Apicoplast and RBC- Red blood cell. Source**:** Krishna et al.

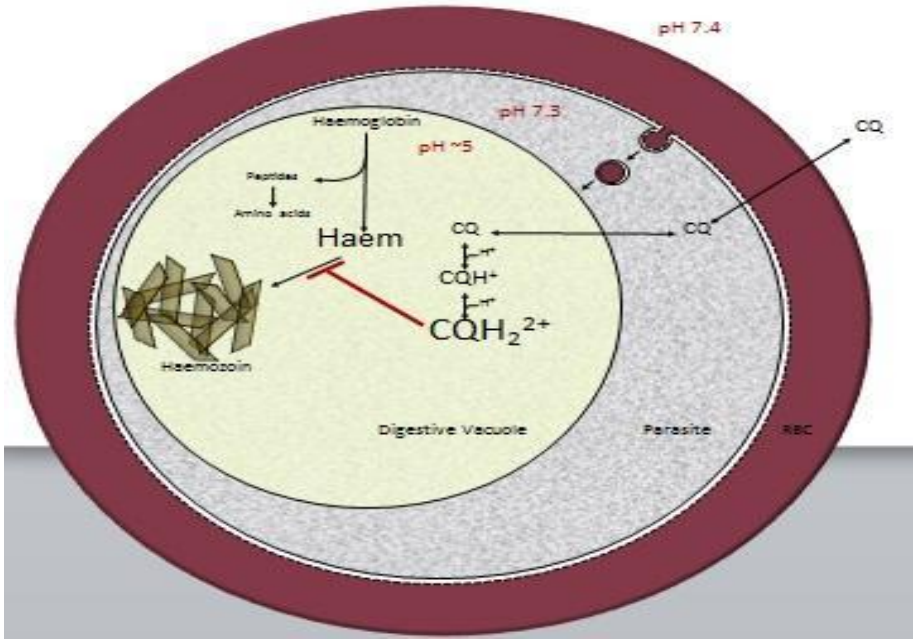

Figure 2: Effect of chloroquine on haemin polymerization; the molecular mechanism of chloroquine action.

However, in this assay, 0.02g of bovine haemin (Sigma-Aldrich) was weighed and dissolved in 2 ml dimethylsulfoxide (DMSO) (Sigma-Aldrich) to ensure the haemin powder is completely dissolved, after which 3 ml distilled water was added to make up 5 ml of 6.5 mM haemin solution (i.e. 40% DMSO).

24.6g of 3 M sodium acetate (Hopkin and Williams) was freshly dissolved in 100 ml distilled water (3M Solution), and 2.5ml of 17.4M glacial acetic acid (Fischer Scientific, UK) was transferred to a 10 ml tube, then 0.2g chloroquine and sulfadoxine (Sigma-Aldrich) were freshly dissolved in 1ml distilled water and 1ml (100% DMSO) respectively. Furthermore, 0.8g of sodium hydroxide (NaOH) (Sigma-Aldrich) was weighed and dissolved in 100 ml distilled water (0.2 M NaOH solution). These reagents were used in this assay to prepare the reaction mixture and demonstrate the conversion of heme to β-haematin.

To make the reaction mixture, 100 µl of the freshly prepared haemin solution was pippeted into 1.5 ml eppendorf tube, then 200 µl of 3 M sodium acetate solution, 50 µl of 17.4 M glacial acetic acid and 50 µl of drug (Chloroquine, positive Control), Sulfadoxine (Negative control) or water (control) was finally added to make a final reaction mixture of 400 µl (pH 4.8 essential for β-haematin formation). Each tube was prepared in duplicate in the order stated as recommended by Tekwani andWalker (2005).

The reaction mixture was gently mixed and incubated at 70 ^{o}C for one hour or 37 ^{o}C for 24 hours, after which the tubes were removed and centrifuged at 13000 revolutions per minute for 15 minutes. The supernatants were discarded and the pellets was dissolved in 100% DMSO and centrifuged further at 13000g for 5 minutes. The pellets were repeatedly washed three to five times, until a clear supernatant is obtained

As previously reported, most assays involve separation of the β-haematin pellet by centrifugation and sequential washing with water, Tris-HCl/SDS solution and alkaline bicarbonate solution or DMSO (Tekwani and Walker, 2005), this assay uses 40% DMSO, 100% DMSO or 25% Sodium dodecyl sulphate (SDS) (Sigma-Aldrich) in an effort to optimize and select for a high throughput method.

Finally, the clear supernatant was discarded and the formed pellet which could be β-haematin was resuspended in 1 ml 0.1M NaOH, the only solvent which can dissolve β-haematin by breaking the iron-carboxylate bond and conversion of β-haematin to heme (Tekwani and Walker, 2005).β-haematin was then quantified spectrophotometrically as heme equivalents.

Spectrophotometric Quantification of β-haematin

Prior to taking the absorbance reading, an aliquot, 1ml of 0.1M NaOH used in dissolving the formed pellet was transferred into a plastic cuvette (Fischer Scientific, UK) and, used as the blank measurement. Later, an aliquot, 200 µl of the resuspended β-haematin solution was further diluted in 800 µl of 0.1M NaOH, and finally transferred into a UV plastic cuvette (Fischer Scientific, UK) and the absorbance at 405 nM was read in a spectrophotometer to quantify the β-haematin solution as heme equivalents. Where absorbance reading were found to be too high (greater than 2), 800 µl of 0.1M NaOH was transferred into a cuvette and 200 µl from the previous cuvette was diluted further and the above step was repeated until a reading (less than 2) is obtained.

Microtitre based assay for quantification of β-haematin

The method previously described by Basilico et al. (1998) was tested and validated. In this assay, the haematin polymerization inhibitory activity of chloroquine and emetine dihydrochloride hydrate was tested using a 96 well microplate (Sterlin limited, UK). 50 µl of 6.5 mM haemin-chloride previously dissolved in 40% DMSO was distributed to 15 wells, 100 µl of 3 M sodium acetate were then added. Haemin polymerization was initiated by adding 25 µl of 17.4 M glacial acetic acid and finally different doses (16 mg/ml, 8 mg/ml, 4 mg/ml and 2 mg/ml) of the test compound (Chloroquine and Emetine dihydrochloride hydrate) were added to triplicate test wells and 25 µl of water to triplicate control wells. The suspension was incubated at 37^{o}C for 24 hours to allow complete polymerisation, conforming a previous report by Tekwani and Walker (2005), that incubation of haemin in glacial acetic acid for 18-24 hours yields formation of β-haematin. Plates were then centrifuged at 3500g for 15 minutes, and the supernatant containing soluble fraction of unprecipitated material was collected. 200 µl of DMSO was added to resuspend the pellets and remove the unreacted haemin. Plates were further centrifuged at 3500g for 15 minutes, and the DMSO soluble fraction was further collected. The final pellet obtained suspected to contain a pure precipitate of β-haematin was dissolved in 0.1 M NaOH for spectrophotometric quantification.

RESULTS

An *in vitro* assay was performed to assess the abilities of chloroquine, sulfadoxine and emetine dihydrochloride hydrate to inhibit β-haematin formation. In the assays, haematin was allowed to form β-haematin under acidic conditions.

Table 1. Effect of different washing steps with different washing solutions.

Washing Solution	Absorbance (405 nM)
25% SDS	1.854
40% DMSO	1.734
100% DMSO	1.449

Table 2. Dose response data obtained from the absorbance reading following treatment with chloroquine and sulfadoxine after haemin polymerization**.**

Dose Concentration (mg/ml)	Chloroquine Absorbance (405 nM)	% Inhibition Chloroquine	Sulfadoxine Absorbance (405 nM)	% Inhibition Sulfadoxine	Control
1	0.715	41.1	1.189	2.05	1.313
2	0.355	70.75	1.153	5.02	1.115
4	0.211	82.62	1.035	14.74	**1.214**
8	0.149	87.7	1.011	16.72	
16	0.117	90.36	0.749	38.3	

Although the technique previously described by Basilico et al. (1998) uses a 96 well U bottomed micro plate, with each well receiving 200 µl of assay mixture, this experiment modifies it by increasing the volume to 400 µl in a 1.5 ml tube. In the same vein, heme (4 mM) used in the previous experiment was dissolved in 0.1 M NaOH, while here; heme (6.5 mM) was dissolved in 40% DMSO.

Different washes using 40% DMSO, 25% SDS and 100% DMSO were tested. In all the trials, absorbance was found to be high in washes with 25% SDS and 40% DMSO (Table 1) while the absorbance rate was lower in washes with 100% DMSO. This method was aimed at determining the effect of different washing steps with different solutions. 100% DMSO was chosen for the subsequent experiments because of its low absorbance and ready to use.

A dose response of chloroquine and sulfadoxine was tested using the same method described by Basilico et al., (1998), and percentage inhibition was calculated using the following formula;

Percentage Inhibition = (Normal activity - Inhibited activity/Normal activity) × 100.

The dose response data obtained for chloroquine and sulfadoxine are presented in Table 2.

As expected, the highest dose (16 mg/ml) of chloroquine cause high percentage inhibition (90%), with the rate of inhibition decreasing as the dose decreases. However, despite the inhibition (38%) caused by the highest dose (16 mg/ml) of sulfadoxine, the percentage inhibition was far less than that caused by the lowest dose (1 mg/ml) of chloroquine (41%). It is not surprising that sulfadoxine does not inhibit haemin polymerization, as numerous literatures report that sulfadoxine act on the folate pathway, hence the reason for its use as a negative control.

The data presented in table 2 was used to plot a graph of percentage inhibition against the different doses (mg/ml) see figure 3.

Microtitre based assay for quantification of β-haematin

Following the success of the previous modification, the exact protocol used in the original assay described by Basilico et al. (1998) was tested and validated. However, the original assay uses a final volume of 200 µl per well (Table 3; Figure 4), a little modification was made in these experiment. Apart from the 200 µl used per well, the experiment was further optimized by reducing the final volume to 100 µl per well (Table 4; Figure 5).

After incubation at 37^{o}C for 24 hours, plates were centrifuged as described earlier, and the supernatants were collected for quantification of un-polymerized haemin (fraction 1). Pellets were dissolved in DMSO, washed and supernatant collected as DMSO soluble (fraction 2) after which pellets were resuspended in 0.1 M NaOH (Fraction 3). All fractions were further diluted in 0.1 M NaOH and the amount of haematin was quantified spectrophotometrically from

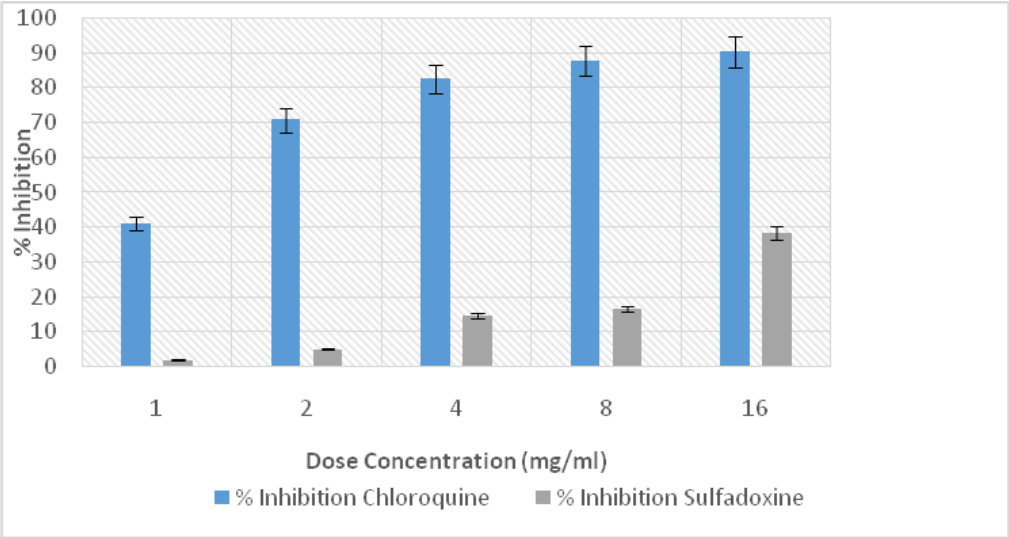

Figure 3. The inhibitory effect of chloroquine and sulfadoxine on polymerization of haemin to β-haematin.

Table 3. Haemin concentration determined from absorbance (405 nM) in un-polymerized (Fraction 1), DMSO soluble (Fraction 2) and NaOH soluble (Fraction 3) haemin. For the 200 µl final reaction volume.

Dose Concentration(mg/ml)	Absorbance (405 nM) 200 µl		
	Fraction 1	**Fraction 2**	**Fraction 3**
2	0.13	0.642	2.759
4	0.073	0.737	3.692
8	0.089	0.507	3.455
16	0.117	2.904	2.221
Control	0.077	0.827	3.412

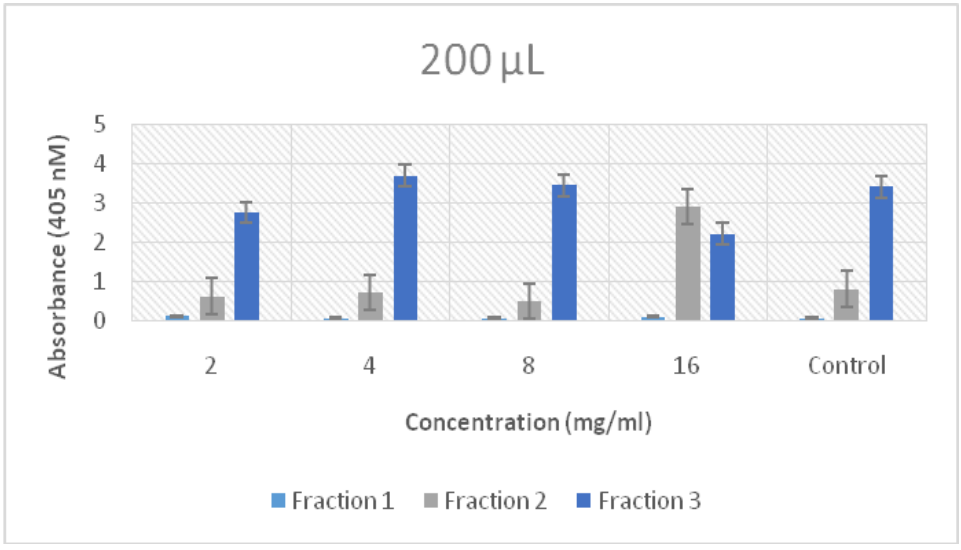

Figure 4. Haemin polymerization inhibitory activity in the presence of different doses of chloroquine.

Table 4. Haemin concentration determined from absorbance (405 nM) in Un-polymerized (Fraction 1), DMSO soluble (Fraction 2) and NaOH soluble (Fraction 3) haemin. For the 100 µl reaction volume per well.

Dose Concentration(mg/ml)	Absorbance (405 nM) 100 µl		
	Fraction 1	Fraction 2	Fraction 3
2	0.267	1.044	4.094
4	0.431	2.784	2.793
8	0.14	3.286	1.672
16	0.083	2.52	0.391
Control	0.551	0.872	3.848

Table 5. Rate of inhibition caused by chloroquine on the NaOH soluble fraction when 100 µl reaction volume was used per well.

Dose Concentration(mg/ml)	Absorbance (405 nM)	% Inhibition
	Fraction 3	
2	4.094	-6
4	2.793	27
8	1.672	64
16	0.391	90
Control	3.848	

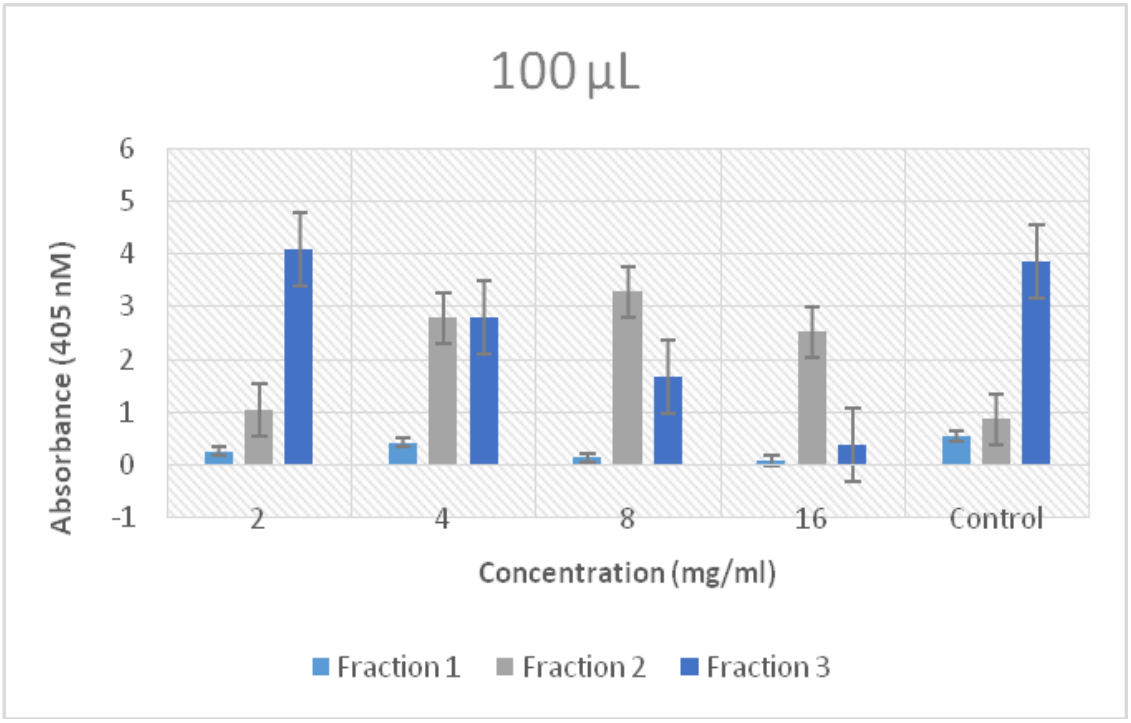

Figure 5. Haemin polymerization inhibitory activity in the presence of different doses of chloroquine.

Table 5. Rate of inhibition caused by chloroquine on the NaOH soluble fraction when 100 µl reaction volume was used per well.

Dose Concentration(mg/ml)	Absorbance (405 nM)	% Inhibition
	Fraction 3	
2	4.094	**-6**
4	2.793	**27**
8	1.672	**64**
16	0.391	**90**
Control	3.848	

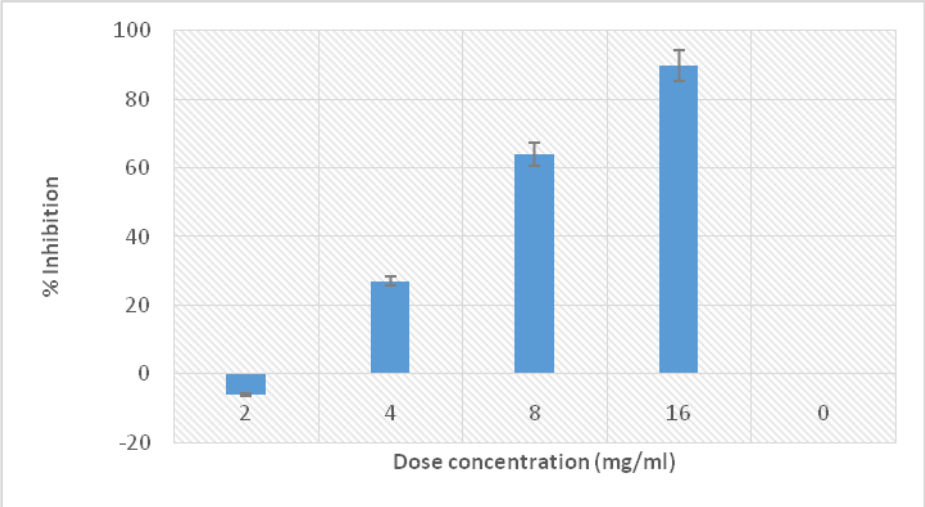

Figure 6. Dose response of chloroquine for NaOH soluble fraction (fraction 3) in the 100µl final reaction volume.

Table 6. Average absorbance reading obtained from triplicate wells for inhibition of β-haematin formation at different doses and concentration following treatment with chloroquine, sulfadoxine, emetine and DMSO.

Dose (mg/ml)	Chloroquine (mg/ml)	Sulfadoxine (mg/ml)	DMSO	Emetine (nM)
2	0.064	0.818	5.577	1.492
4	0.072	1.728	4.145	4.799
8	0.177	2.323	4.757	1.583
16	0.248	3.838	3.062	3.232
Control	0.411	1.074	1.009	1.796

the absorbance of haematin in 0.1M NaOH at 405 nM (table 3). However, the 3rd fraction show high absorbance at 405 nM than the 1st and 2nd fractions.

Figure 5 is a graphical representation of the inhibitory effect of chloroquine on haemin polymerization. Although the absorbance obtained from dilution of the three fractions (table 4) was used to plot the graph, fraction 3 show high absorbance at low drug concentration (2mg/ml) but low percentage inhibition (< 1%) at the same concentration, while low absorbance was seen at high drug concentration (16mg/ml) but high percentage inhibition (90%) at the same concentration.

From the absorbance reading obtained when 100 µl reaction volume was used per well, a percentage inhibition was calculated only for the NaOH soluble fraction (fraction 3) using the formula stated above, and result show high inhibition

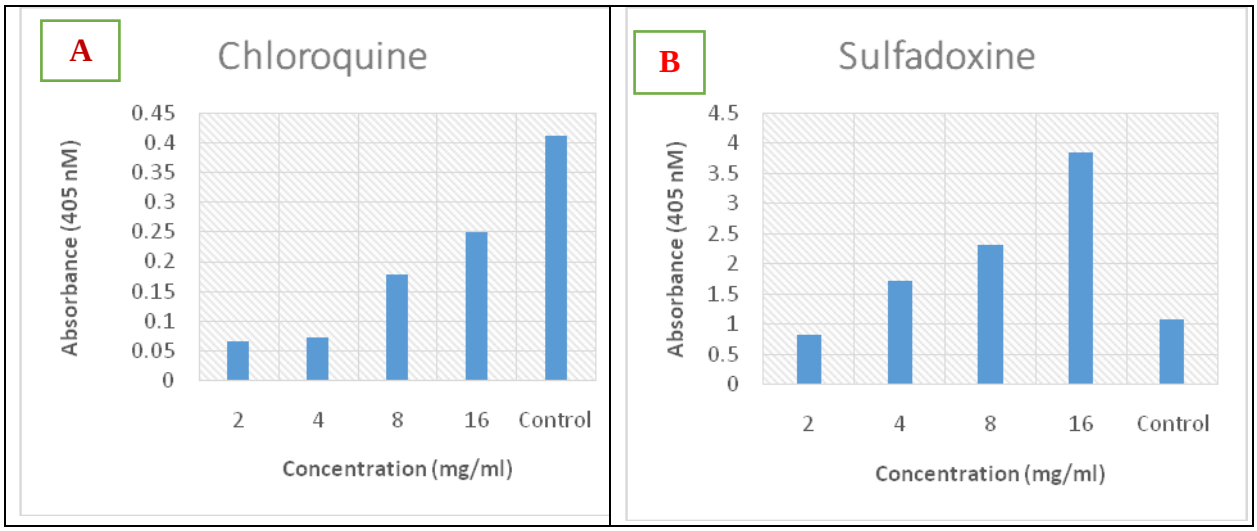

Figure 7. Dose response of chloroquine (a) and sulfadoxine plotted against the absorbance reading.

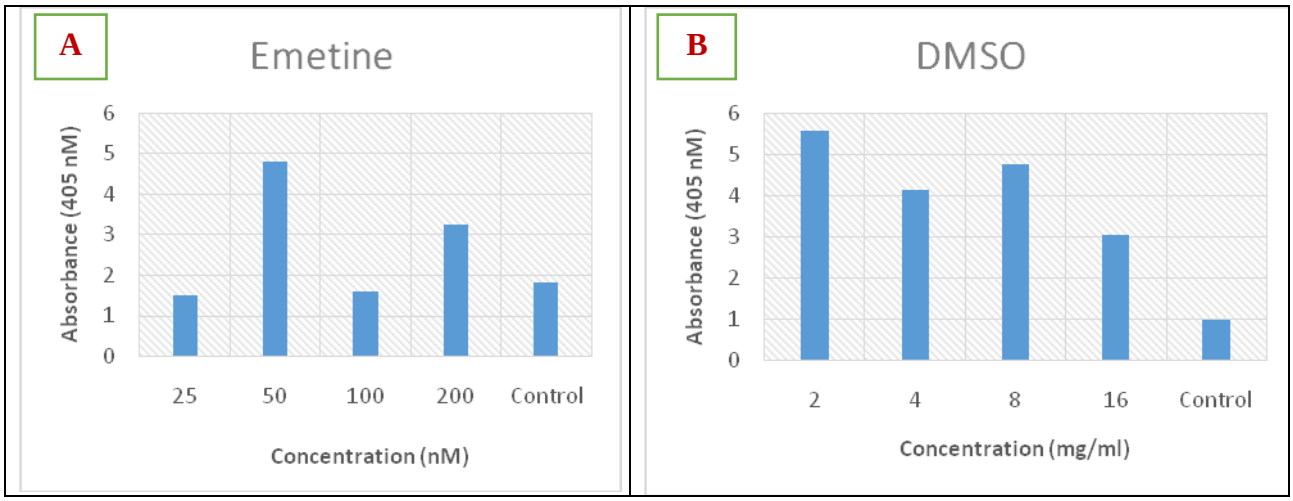

Figure 8. Dose response of Emetine and different concentration of DMSO plotted against the absorbance reading.

(90%) at higher dose and very low (< 1%) inhibition at the lower dose (Table 5). However, a graphical representation of these data is presented in figure 6.

Following protocol validation, the experiment was repeated with the inclusion of emetine dihydrochloride hydrate and sulfadoxine as well as chloroquine. The effect of DMSO on haemin polymerization was also tested (Table 6). Results obtained from the experiment shows high absorbance at higher doses of chloroquine and sulfadoxine (Figure 7a and 7b) and emetine dihydrochloride hydrate (Figure 8a). Although the absorbance obtained at 50 nM (the IC_{50} for emetine) was higher than the highest dose, it is possible that an inconsistency in pipetting had occurred during the experiment. While the result obtained for DMSO (Figure 8b) was fluctuating, there is a possibility of effect of pipetting during the experiment.

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DISCUSSION

It is well-known that malaria parasite reside in the red blood cells of their mammalian host. During the intra erythrocytic development of the parasite, haemoglobin, the oxygen carrying pigment is ingested by the parasite and used as its

major protein source. As the parasite develops further, ~80% of haemoglobin in the RBCs are degraded by the developing trophozoites. Ingested haemoglobin are broken down to haem (iron rich molecule) and globin molecules respectively. Globins are converted to amino acid for use by the parasite, while haem is toxic, and when allowed to accumulate, can result in membrane lysis, that eventually leads to parasite death. To overcome the toxicity of free haem, malaria parasites convert the toxic haem to a non-toxic haemozoin otherwise called the malaria pigment.
Due to the lack of available vaccines for malaria, and virtually all trialled vaccine candidates including the leading RTS,S have failed or have low efficacy during trial, drugs remains the mainstay for malaria treatment. Various drugs have been used in the treatment of malaria, each acting on a specific pathway. The quinolines act on the haemin polymerization pathway, antifolates act on the folate pathway while the specific pathway for artemisinins remains unclear, although different pathways have been proposed for artemisinins.

In this study, the in vitro antimalarial efficacy of emetine dihydrochloride hydrate was tested in an effort to determine the pathway to which it act in the malaria parasite, as previous finding by Matthews et al., (2013), suggest that emetine has an antimalarial effect on the multidrug resistant strain (K1) of *P. falciparum*. However, emetine, derived from the root of *Carapichea ipecacuanha* (Ipecac®) was formerly an antiprotozoal drug used in the treatment of invasive intestinal amoebiasis and amoebic liver abscess (Matthews et al., 2013). Prior to testing the inhibitory effect emetine on haemin to β-haematin polymerization, the β-haematin (haemozoin or malaria pigment) formation assay was optimized and the inhibitory effect of chloroquine (known to prevent the formation of β-haematin) and sulfadoxine was tested.

Results obtained from this study, according to the method previously described by Auparakkitanon et al. (2003) was not successful possibly because drug was added to haemin solution before the initiation of haemin polymerization by addition of sodium acetate and glacial acetic acid. In addition, making the assay mixture in light condition, or possibly making DMSO solution before the addition of haemin powder might likely be the result of reaction failure. Because haemoglobin digestion and haem detoxification occur in the acidic food vacuole of malaria parasite, Tripathi et al. (2004) suggest that *in vitro* haemin polymerization should be initiated in acidic medium. However, in order to overcome reaction failure, further preparations were always in the dark, haemin powder was dissolved in DMSO before the addition of water and haemin polymerization was initiated in acidic medium before drug is added.

Further reaction preparations in the dark, dissolving haemin powder in DMSO before the addition of water and initiation of haemin polymerization by addition of sodium acetate and acetic acid before the addition of drug yield the expected result. The in vitro β-haematin inhibitory effect of chloroquine found in this study goes in accordance with the results of Baelmans et al. (2000). At higher doses, high percentage inhibition (90%) of β-haematin formation by chloroquine was seen, which also corresponds with the results of Basilico et al. (1998), who reported that β-haematin/haematin aggregates were inhibited by 90% in the presence of chloroquine diphosphate at higher concentrations. Because chloroquine diphosphate was used in this assay, it is suspected that phosphate is responsible for the inhibitory effect of β-haematin formation caused by chloroquine diphosphate as Baelmans et al. (2000) reported that phosphate and chloride salts show inhibitory effects on the haemin to β-haematin polymerization by 85% and 97% respectively, but no inhibition was observed with chloroquine sulphate. Nonetheless, further investigation suggest that the inhibition may perhaps not be due to change in ionic strength as the assay was done in molar concentrations of acetate.

Although, the previous assay was done in a 1.5 ml eppendorf tube and yields the expected result, further optimization of the same protocol in a microtitre plate was performed. This is because; haemin polymerization to form β-haematin in acidic condition in microplate was thought to offer several advantages over tubes for screening compounds. According to Basilico et al. (1998), the advantages of the use of microplate include the ability to assay samples and drugs in triplicates or even quadruplets, increasing the assay throughput, ability to monitor the amount of precipitate spectrophotometrically using microplate readers as well as the use of multichannel pipetters, plate shakers and washers and table top centrifuge for dispensing samples and recovering supernatants. Moreover, reaction volumes are reduced, less compounds are needed and drugs whose stock is very limited or expensive can be tested.

When 100 µl final reaction volume was used in a microplate, high absorbance was observed for the NaOH soluble fraction (fraction 3), which is strongly connected with the single dilution (one in ten) of pellets in 0.1 M NaOH. Further dilution might yield low absorbance. Despite the high absorbance obtained, the highest dose of chloroquine (16 mg/ml) result in 90% inhibition (table 6), which is in consonance with the result seen when 1.5 ml tube was used. This suggest that as low as 100 µl final reaction volume can be used for β-haematin inhibitory assays, offering advantages of performing multidrug screening at a time and ability to increase assay throughput.

Furthermore, the same protocol used above was repeated with the inclusion of emetine dihydrochloride hydrate and sulfadoxine. The high absorbance obtained at the higher doses of both drugs and low absorbance at lower dose indicates that the reaction was not successful. Even though, similar conditions used in the previous assay were applied here, a possible inconsistency in pipetting during the experiment might be the consequence of the result seen. However, it still remains inconclusive on whether emetine inhibit haemin polymerization or not, unless the assay is repeated several times and no inhibition is seen. But for sulfadoxine, many literatures suggest its inhibitory activity on the folate

pathway and not haemin to β-haematin pathway (Nzila, 2006 b; Muller and Hyde, 2013). It is therefore not surprising that inhibition was not seen, hence the reason for its use as the negative control.

CONCLUSION

The present study was designed to determine the β-haematin inhibitory activity of emetine dihydrochloride hydrate, with the use of chloroquine diphosphate and sulfadoxine as positive and negative controls respectively.
Results obtained justify the inhibitory activity of chloroquine on the haemin polymerization pathway, but the β-haematin inhibitory activity of emetine remains inconclusive. Whilst the study did not confirm the in vitro β-haematin inhibitory activity of emetine, more research is required to determine the exact pathway to which emetine act upon in the malaria parasite and, more broadly, further investigation into its inhibitory activity on the haemin polymerization pathway is needed before a conclusion is drawn on whether or not it target that pathway.

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