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Research Article

***Plasmodium falciparum*: Assessment of Selectivity of Action of Chloroquine, *Alchornea cordifolia*, *Ficus polita*, and Other Drugs by a Tetrazolium-Based Colorimetric Assay**

Nana Kofi Ayisi,^{1,2} Regina Appiah-Opong,³ Ben Gyan,⁴ Kwasi Bugyei,⁵ and Fred Ekuban³

¹ Virology Department, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana (UG), Legon, Ghana

² Department of Pharmacology, School of Medical Sciences, University of Cape Coast, Cape Coast, Ghana

³ Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana (UG), Legon, Ghana

⁴ Department of Immunology, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana (UG), Legon, Ghana

⁵ Department of Pharmacology, University of Ghana Medical School, Korle Bu, Accra, Ghana

Correspondence should be addressed to Nana Kofi Ayisi, nana.ayisi@yahoo.co.uk

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A tetrazolium-based colorimetric selective assay (MTT-based CSA) was developed to assess the selectivity of antimalarial drugs. This *in vitro* assay, unlike all others, measures the ability of drugs to indirectly protect red blood cells (RBCs) from *Plasmodium falciparum*-induced destruction. Optimum incubation time and number of cells needed were 5 days and 23×10^6 RBCs per well, respectively. A parasitemia range of 0.375% to 3% was found to be suitable for this assay. The MTT-based CSA determined anti-*P. falciparum* strain DD2 activity of chloroquine at a higher 50% effective concentration (EC₅₀) value (21.0 µg/mL) than the isotopic microtest (10.0 µg/mL). Artesunate and oxytetracycline achieved 90% effect against DD2 with minimal or no toxicity to RBCs. Against chloroquine sensitive strain 3D7, chloroquine and *Alchornea cordifolia* had EC₅₀ values of 0.025 µg/mL and 4.9 µg/mL respectively, and selective index (SI) values of >2,000 and >69.4 µg/mL, respectively.

1. Introduction

The threat of malaria has continued to plague millions of people living in most tropical countries despite numerous strategies to bring this disease under control. Chloroquine used to be the first drug of choice for the treatment of *P. falciparum* infections in Ghana and many African countries, but the emergence of drug-resistant strains has necessitated intensive search for new drugs [1–3]. Presently, Artesunate-Amodiaquine and Artesunate-Lumefantrine combinations are first-choice drugs in Ghana. The standard World Health Organization (WHO) method for testing new antimalarial drugs involves tedious microscopic parasite counting method (MPCM). Other *in vitro* methods are haem polymerization inhibition assay (HPIA) [4], lactate dehydrogenase assay (LDHA) [5, 6] and the isotopic microtest (IMT) [7]. All these *in vitro* tests measure some activity of the

parasite and the endpoints are directly parasite related. Destruction of red blood cells (RBCs) is the end result of malarial infection. None of the available *in vitro* assays uses RBCs-related activity to determine endpoints. Furthermore, the available *in vitro* assays do not test for drug selectivity unless a separate assay on RBC toxicity is done. In this paper, we report on a tetrazolium-based colorimetric selective assay (MTT-based CSA) that uses indirect protection of RBCs from parasite destruction as an endpoint for measuring the effect of drugs on parasites and also determines the possible drug toxicity to the RBCs.

2. Materials and Methods

2.1. Drugs and Plant Extracts. Chloroquine diphosphate (Sigma CQ) was obtained from SIGMA, St Louis, Mo, USA. Also tested in comparison were formulated chloroquine

(Troge Medical GmbH, Hamburg, Germany), formulated chloroguanide (proguanil) (Zeneca Pharmaceuticals, UK), formulated artesunate (Guilin Pharmaceutical Works, China), and formulated oxytetracycline (Bimeda, Ireland) purchased from a local pharmacy. Plant extracts GHX-26F and GHX-6L are aqueous extracts from the fruits of *Alchornea cordifolia* and the leaves of *Ficus polita* respectively. The aqueous extracts were freeze-dried. Both plants are effective against human immunodeficiency virus in vitro [8] and are suspected to have antiprotozoal activities. All drugs and plant extracts were dissolved in culture medium and filtered through sterile 0.45 μm filters.

2.2. Parasites. Strains 3D7 (chloroquine sensitive) and DD2 (chloroquine resistant) of *P. falciparum* were obtained from Dr. Jorgehn Kurtzhals, Center for Medical Parasitology, University of Copenhagen, Denmark.

2.3. Cell Cultures. *P. falciparum* infected and uninfected red blood cells (RBCs) were cultured in RPMI 1640 (Gibco, Parsley, UK) supplemented with 20 mmol/mL L-glutamine (Sigma-Aldrich Co. Ltd., UK), 20 $\mu\text{g}/\text{mL}$ gentamicin and 2.5 $\mu\text{g}/\text{mL}$ fungizone (Gibco, Parsley, UK), 10% albumax II (Gibco BRL, Grand Island, NY, USA), or 10% normal human AB serum (Sigma-Aldrich Co. Ltd., UK). The cultures were flushed with a gas mixture consisting of 3% O_2 , 6% CO_2 , and 91% N_2 , sealed and incubated at 37°C. For experiments in 96-well microtitre plates, the cultures were put in a desiccator with a lit candle and nearly completely covered. As soon as the candle went off, the desiccator was completely closed.

2.4. Determination of Optimum RBC Number and Incubation Time. *P. falciparum*-strain-3D7-infected (1% parasitemia) and uninfected cultures were incubated as described above and observed daily for color differences between the two cultures. The parasite-infected culture started becoming visually pale from day 3. The difference in color between the infected and uninfected cultures increased up to day 5. There was no change in the observed paleness of the infected culture from day 5 to day 6. Day 5 was therefore considered as the probable optimum incubation time for visually determined optimum parasite-induced damage to RBCs. The number of RBCs in culture that would give maximum difference between optical densities (ODs) of uninfected and *P. falciparum*-infected RBCs was then determined as follows. The numbers of healthy RBCs in uninfected and parasite-infected stock cultures were determined by the trypan blue exclusion method and adjusted to be the same per milliliter. Twofold serial dilutions of stock cell cultures (uninfected or 5% infected) were made and 100 μL was put in wells of 96-well microtitre plates that already contained 100 μL of growth medium bringing the total volume per well to 200 μL . Each cell dilution was put in six replicate wells. The cultures were incubated in a desiccator as described above and after 5 days of incubation, cell viability was determined by the MTT-based colorimetric method as previously described [9]. Twenty microliters (20 μL) of MTT (7.5 mg/mL) was added to each well and the plates incubated at 37°C for another 2 h. One hundred and fifty microliters (150) μL of medium was then carefully

taken from each well and discarded. Two hundred microliters (200 μL) of 10% Triton X-100 in acidified isopropanol was added to each well in order to dissolve any formed formazan. With the aid of a multichannel micropipettor, the contents of each well were mixed several times, and the plates kept at room temperature in the dark for 24 h. The optical densities of the wells were read by Emax Precision Microplate reader (Molecular Devices Corporation, Menlo Park, Calif, USA) at 565 nm and 690 nm. Readings at 690 nm were automatically subtracted from readings at 565 nm by the microplate reader. The experiment was repeated once. The averages of all readings were recorded for each dilution. A graph of cell number versus differences in OD readings of uninfected and infected wells was then plotted to determine the optimum cell number that will give the highest OD reading difference between uninfected and infected wells.

2.5. Determination of Optimum Parasitemia. Approximately, 23×10^6 cells per well were determined to give optimum difference in OD readings between uninfected and *P. falciparum* infected cells in the above experiment. Twenty-three million (23×10^6) cells infected at various parasitemia ranging from 0% to 6% were put into wells of 96-well microtitre plates and incubated at 37°C for 5 days. Each parasitemia was tested at 6 replicates. The cells were processed for MTT-based colorimetric assay as described above. The average OD readings at various levels of parasitemia were subtracted from the average OD reading of control uninfected wells and the values plotted against % parasitemia.

2.6. Tetrazolium-Based Colorimetric Selective Assay (MTT-Based CSA). From the results of the foregoing experiments, optimum time of incubation, optimum number of RBCs, and optimum parasitemia were determined to be 5 days, 23×10^6 cells per well, and 3% parasitemia, respectively. The drug inhibition experiments therefore utilized these three values in the testing of chloroquine, chloroguanide (proguanil), artesunate, oxytetracycline, and two plant extracts against *P. falciparum* infection in RBC cultures. In the case of Sigma CQ, 1.5% parasitemia was used. Drug or plant extract was serially diluted, and a 100 μL of each dilution was put into six wells of 96-well microtitre plates. One hundred microliters (100 μL) of uninfected RBCs was added to three replicate drug dilutions. One hundred microliters (100 μL) of infected RBCs was added to three other replicate drug dilutions. Drug-medium controls in the top and bottom outer wells were included in the experiments. The cultures were incubated in a desiccator as described in the previous experiments. The MTT-based colorimetric test was then performed on the cultures. The percent protection of RBCs from malaria parasite destruction by different drugs or plant extracts concentrations were determined by the following formula:

Percent RBC Protection

$$= \left\{ 1 + \frac{(\text{ODT})_{\text{Pf}} - (\text{ODT})_{\text{mock}}}{(\text{ODC})_{\text{mock}} - (\text{ODC})_{\text{Pf}}} \right\} \times 100, \quad (1)$$

where $(\text{ODT})_{\text{Pf}}$ is the average OD measured for a given concentration of the test drug in *P. falciparum*-infected

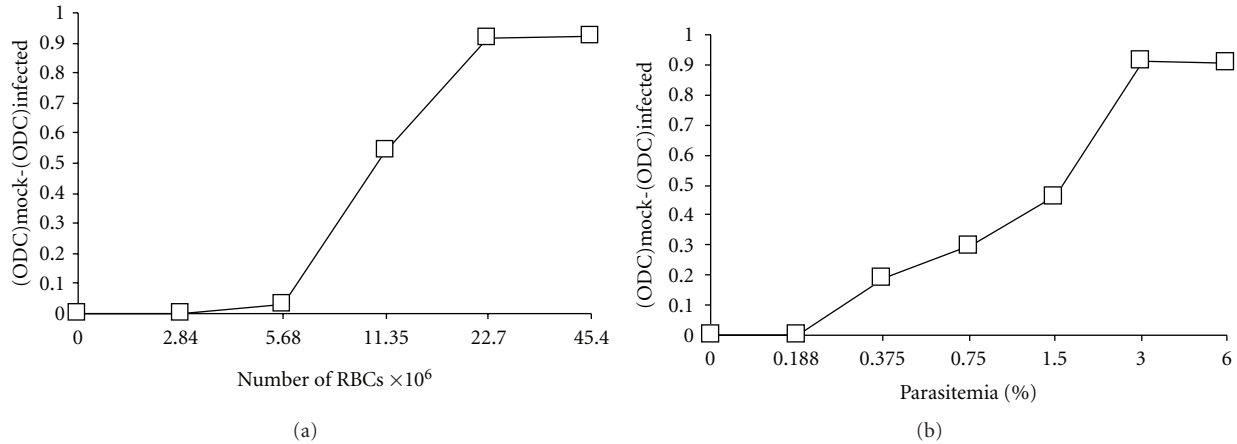


FIGURE 1: Effects of number of red blood cells (a) and parasitemia (b) on *P. falciparum* cytopathicity, that is, optical density of mock-infected RBCs minus optical density of parasite-infected RBCs [(OD)_{mock} - (OD)_{infected}]. Infection was done with strain 3D7.

RBCs; (ODT)_{mock} is the average OD measured for a given concentration of test drug in uninfected RBCs; (ODC)_{mock} is the average OD measured for the control untreated uninfected RBCs; (ODC)_{pf} is the average OD measured for the control untreated *P. falciparum*-infected RBCs. The average percent RBC protection values for each three replicates were plotted against the concentrations of drugs and the concentrations needed to achieve 50% and 90% RBC protection, that is, effective concentrations (EC₅₀ and EC₉₀) determined by regression analysis. The percent RBC survival determined the extent of drug toxicity and was calculated by the following formula:

$$\text{Percent RBC Survival} = \frac{(\text{ODT})_{\text{mock}} - (\text{ODT})_{\text{cf}}}{(\text{ODC})_{\text{mock}} - (\text{ODC})_{\text{cf}}} \times 100, \quad (2)$$

where (ODT)_{cf} is the average OD measured for cell-free treated wells; and (ODC)_{cf} is the average OD measured for cell-free untreated wells. These values were plotted against the concentrations of the drugs and the concentrations needed to reduce RBC survival by 50%, that is, 50% cytotoxic concentrations (CC₅₀) determined by regression analysis. Anti-*P. falciparum* indices or selective indices (SI) were then calculated as CC₅₀/EC₅₀.

2.7. Isotopic Microtest (IMT) Assay. The effect of Sigma CQ on *P. falciparum* strain DD2 was tested in the IMT assay concurrently as in the MTT-based CSA. In the IMT assay, the incubation was interrupted after 24 hours for the addition of 0.5 μCi of ³H-hypoxanthine, and incubation continued for additional 24 hours before measurement of ³H-hypoxanthine uptake as published before [7]. The percent inhibition was plotted against concentration of drug and the EC₅₀ and EC₉₀ values determined by regression analysis.

3. Results

3.1. Optimum Incubation Time and Cell Number. By visual examination, 5 days of incubation was found to give

maximum lysis in infected RBCs with very little cell death in uninfected control cultures. Figure 1(a) shows the effect of number of RBCs on the difference in OD readings between infected and uninfected RBCs. The cell number that produced the highest difference in OD readings between infected and uninfected RBCs was about 23 × 10⁶ per well.

3.2. Optimum Parasitemia. Figure 1(b) shows the effect of parasitemia on the ability of RBCs to metabolize tetrazolium to formazan. A parasitemia of 3% caused the optimum damage to RBCs leading to high OD reading difference between uninfected and infected RBCs of 0.92. The lowest parasitemia at which enough damage to RBCs could be detected was found to be 0.375% where the OD reading difference between uninfected and infected RBCs was 0.2.

3.3. Comparative Activities of Drugs and Plant Extracts against DD2 and 3D7 Strains of *P. falciparum*. The antimalarial activity of chloroquine diphosphate obtained from Sigma was tested against strain DD2 in both the isotopic IMT and the MTT-based CSA and the results are given in Figure 2 and Table 1. Sigma CQ was slightly more active in the IMT than in the MTT-based CSA. The IMT unlike the MTT-based CSA, does not include a toxicity test and is therefore not applicable to the determination of selective index of a drug. In the MTT-based CSA, formulated CQ (Figure 3(a) and Table 1) like Sigma CQ had moderate activity against this strain of *P. falciparum*. Proguanil (Figure 4(a)) and plant extract GHX-26F (Figure 3(b) and Table 1) were minimally effective against DD2. Formulated artesunate (Figure 4(b)) and oxytetracycline (Figure 4(c)) were very effective against DD2. Formulated CQ and plant extract GHX-26F were very effective in the MTT-based CSA against chloroquine sensitive 3D7 with SI (CC₅₀/EC₅₀) values of >2,000 and >69.4, respectively (Table 1). Figure 5 is from a separate experiment done for pictures to be taken to demonstrate that the effects of drugs can be observed visually in the MTT-based CSA. Clearly, formulated CQ and GHX-26F were very effective against strain 3D7, whereas GHX-6L had no activity.

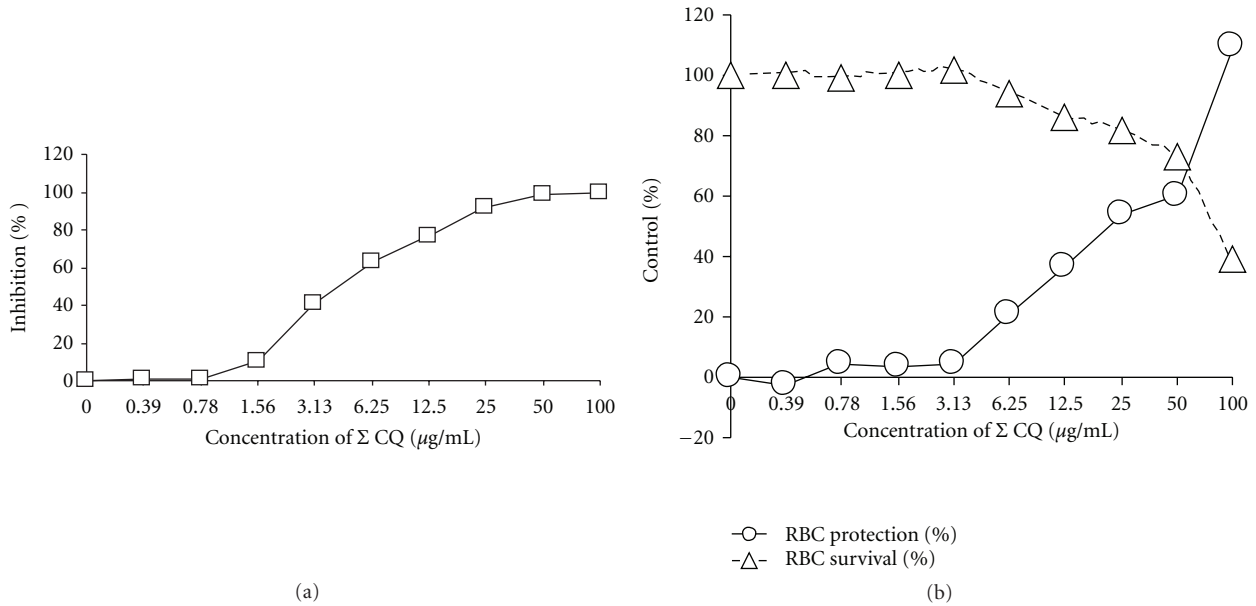


FIGURE 2: Anti-*P. falciparum* activity and toxicity of chloroquine (purchased from Sigma) tested in the IMT (a) and MTT-based CSA (b) using parasite strain DD2.

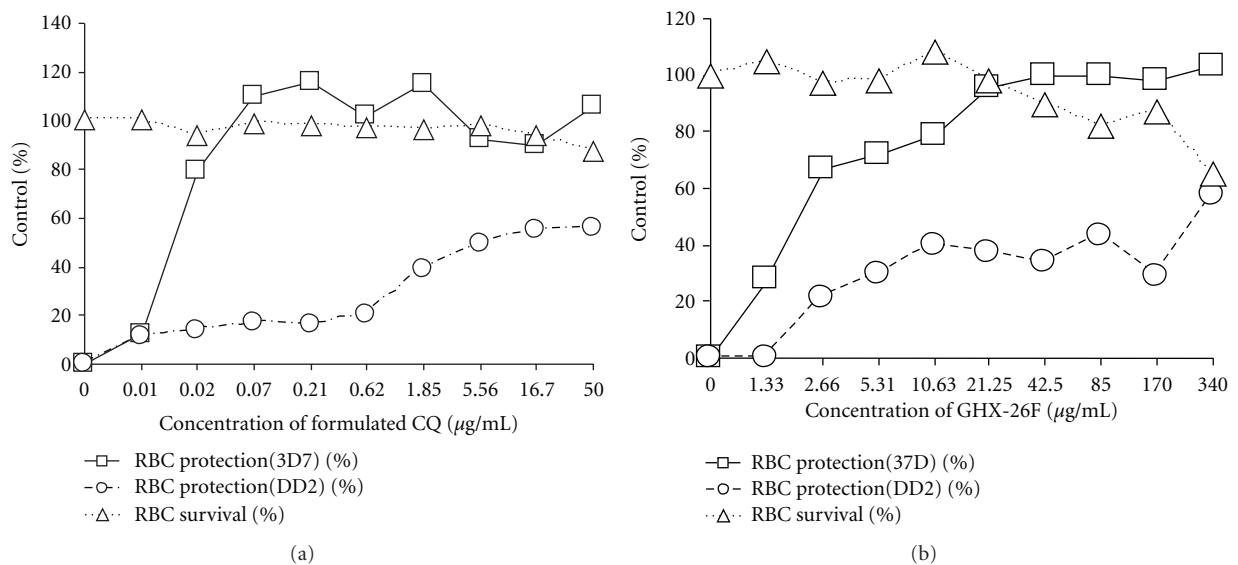


FIGURE 3: Anti-*P. falciparum* activity and toxicity of formulated chloroquine (a) and plant extract GHX-26F (b) tested in the MTT-based CSA using parasite strains 3D7 and DD2.

4. Discussion

Determination of in vitro selectivity is crucial in identifying new compounds with potentials as true antimalarial drugs. There is no available in vitro antimalarial assay system that determines drug selectivity. The term selectivity of action in this paper refers to the extent to which the effect of drug is due to parasite events alone and not an indirect effect resulting from effects on uninfected RBCs. This is very important because a compound may destroy RBCs and therefore prevent malaria parasite replication which will lead

to false-positive results. All available assays measure parasite-related activity. Our MTT-based CSA measures RBC-related activity of metabolism of tetrazolium into formazan. The end result is a measure of the ability of a compound to indirectly protect RBCs from *P. falciparum*-mediated lysis. The formula for calculating the percent RBC protection is the same as published for antihuman immunodeficiency drugs [9]. The formula for calculating the percent RBC survival (toxicity) has however been modified to account for plant extracts and other drugs whose color may increase the background OD reading. The maximum difference in OD values between

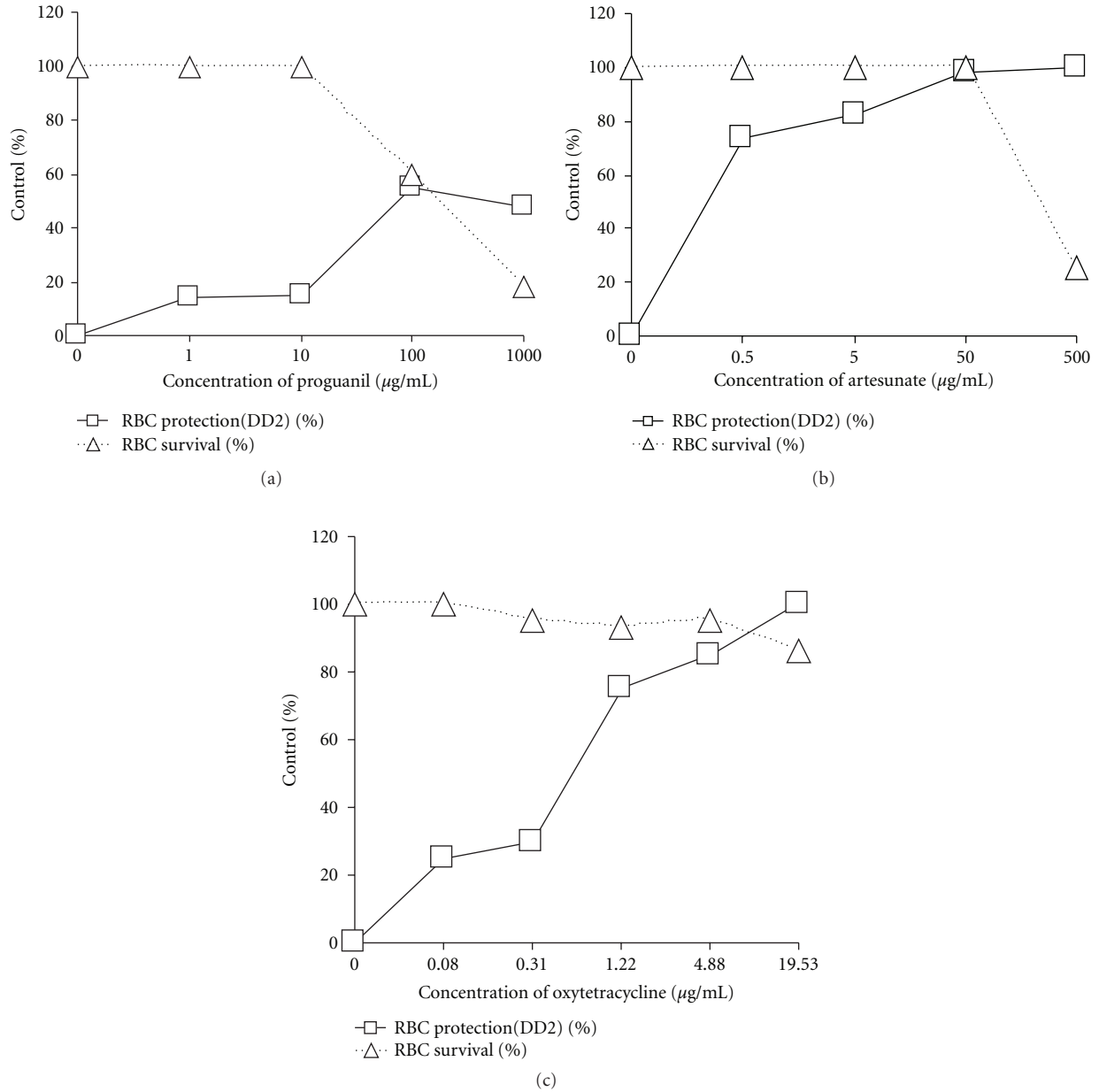


FIGURE 4: Anti-*P. falciparum* activity and toxicity of formulated proguanil (a), artesunate (b), and oxytetracycline (c) tested in the MTT-based CSA using parasite strain DD2.

TABLE 1: Effects of drugs and plant extracts against *P. falciparum* strains DD2 (A) and 3D7 (B) infection in RBCs.

	Drug	EC ₅₀ µg/mL	EC ₉₀ µg/mL	CC ₅₀ µg/mL	SI (CC ₅₀ /EC ₅₀)
(A)	Sigma CQ ^{a*}	10.0	20.4	NA	NA
	Sigma CQ ^{b*}	21.0	77.9	82.7	3.9
	Formulated CQ ^b	32.6	>50 ^c	>50 ^c	>1.5
	GHX-6L ^b	>445 ^c	>445 ^c	>445 ^c	ND
	GHX-26F ^b	272.8	>340 ^c	>340 ^c	>1.2
(B)	Formulated CQ ^b	0.025	0.05	>50 ^c	>2,000
	GHX-6L ^b	>445 ^c	>445 ^c	>445 ^c	ND
	GHX-26F ^b	4.9	16.2	>340 ^c	>69.4

^aTested by IMT, ^bTested by MTT-based CSA, *Parasitemia used was 1.5%. For the other tests, parasitemia used was 3.0%, ^cHighest concentration tested, SI: selective index, NA: not applicable, ND: not determined.

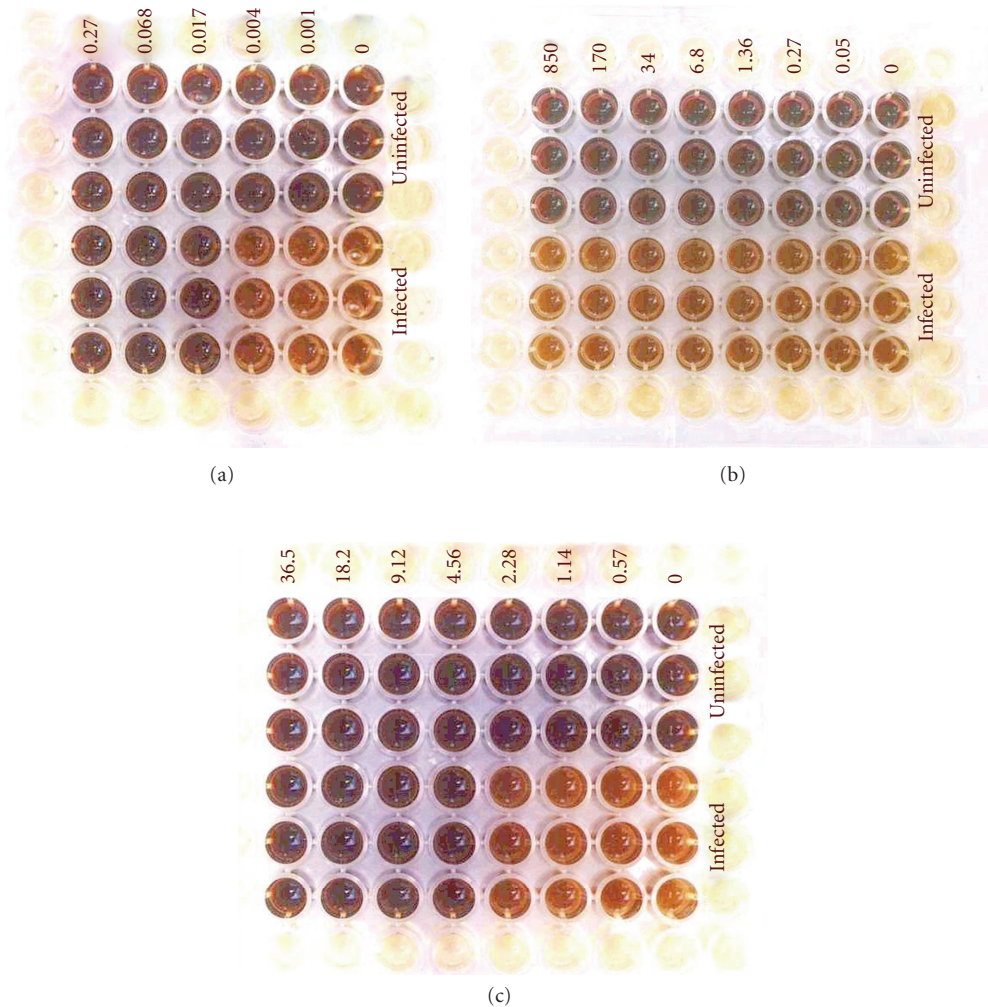


FIGURE 5: Photographs of parasite strain 3D7-infected (bottom three replicates) and -uninfected (top three replicates) RBCs treated with formulated chloroquine (a), plant extract GHX-6L (b), and plant extract GHX-26F (c) after the tetrazolium reaction. Concentrations are given as $\mu\text{g/mL}$.

uninfected and infected RBCs may vary depending on the source of RBCs used as a result of differences in certain genetic factors involved in drug metabolism.

We used a specific number of RBCs instead of a specific hematocrit because the formation of formazan is dependent on healthy and metabolically active RBCs. Even though approximately 23×10^6 RBCs/well was determined to give the maximum difference between OD readings of mock-infected and parasite-infected RBCs, lower numbers of RBCs that give OD reading difference of at least 0.2 can be considered as a minimum requirement (Figure 1(a)). A similar case can be made for the parasitemia where reliable determinations can be made using at least 0.375% parasitemia (Figure 1(b)). Chloroquine diphosphate (SIGMA CQ) was tested in the IMT and MTT-based CSA using 1.5% parasitemia similar to other tests. For the other drugs, 3% parasitemia was used in order to be able to clearly visualize the effect of compounds before and after the addition of tetrazolium without OD reading by microplate reader. The choice of not changing the medium during the 5 days of incubation was to allow for the death of the parasites so that only the metabolic

activity of the RBCs would be determined by the MTT-based assay.

As expected, chloroquine was not very effective against *P. falciparum* strain DD2 when tested in either the IMT or MTT-based CSA. There was very little difference between unformulated chloroquine bought from SIGMA and formulated chloroquine bought from a local pharmacy even though the latter was tested at a higher parasitemia. Proguanil, artesunate, and oxytetracycline were tested just to determine their effectiveness in inhibiting chloroquine-resistant DD2 induced RBC lysis. No attempt was made to find EC_{50} concentrations of these drugs since only four concentrations of each drug were tested. Proguanil needs to be activated to a triazine derivative in vivo to have any significant effect on *P. falciparum* infection [10]. The minimal activity of proguanil observed against chloroquine resistant strain DD2 may be due to the little intrinsic activity of the drug. Artesunate and oxytetracycline were very effective against DD2 at concentrations with very little or no toxicity to RBCs. Artesunate is a peroxide antimalarial drug which is activated by haem and through a series of processes, it is suspected

to yield reactive species capable of alkylating key cellular components and that cause parasite death [11, 12]. Oxytetracycline inhibits protein synthesis and was expected to be effective against DD2 strain as confirmed in our results.

The use of RBC-related activity to determine endpoint drug effect makes it inappropriate to compare our assay with other available in vitro assays. Nevertheless, it would suffice to say that our assay has the advantage of determining the selectivity of drug action. We have used the MTT-based CSA to show that one of two plants suspected to have antiprotozoal activity, that is, *Alchornea cordifolia*, is indeed very effective against chloroquine-sensitive 3D7 with a high-selective index. The development of an in vitro assay that determines the selectivity of action would be even more important for malarial parasite infection in RBCs from people with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency or those with congenital deficiency of nicotinamide adenine dinucleotide (NADH) methemoglobin reductase in whom antimalarial drug toxicity is of paramount concern. One drawback that is common for all in vitro testing is that the potential new drug may need liver enzyme metabolism to the active form. An additional step of incubating the compound with liver extract before testing will remedy this drawback. Whereas existing in vitro antimalarial tests may be faster and reliable for drugs with known toxicity profiles, our MTT-based CSA stands out as providing results on the selectivity of action of potential new antimalarial drugs thereby allowing for early decision making for further development. Finally, using the extent of RBCs destruction to measure the endpoint gives us a much clearer assessment of what is likely to occur in vivo.

5. Conclusions

A tetrazolium-based selective colorimetric assay was found to be suitable in identifying potential new antimalarial drugs. With the inclusion of toxicity studies in this assay, antimalarial indices can be calculated to make sure that new compounds are truly effective against the parasite. The plant extract *Alchornea cordifolia* was shown to be very effective against *P. falciparum* strain 3D7.

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