

A NOVEL DNA-BASED MICROFLUORIMETRIC METHOD TO EVALUATE ANTIMALARIAL DRUG ACTIVITY

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Abstract. This paper describes the development of a novel microfluorimetric assay to measure the inhibition of *Plasmodium falciparum* based on the detection of parasitic DNA by intercalation with PicoGreen[®]. The method was used to determine parasite inhibition profiles and 50% inhibitory concentration values of known or potential anti-malarial drugs. Values for parasite inhibition with known anti-malarial drugs using the PicoGreen[®] assay were comparable with those determined by the standard method based upon the uptake of ³H-hypoxanthine and the Giemsa stain microscopic technique. The PicoGreen[®] assay is rapid, sensitive, reproducible, easily interpreted, and ideally suited for screening of large numbers of samples for anti-malarial drug development.

INTRODUCTION

Malaria is among the most life-threatening and widespread diseases in the world, causing 250–300 million cases and approximately two million deaths annually.¹ The disease is caused by four *Plasmodium* species (i.e., *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) that are transmitted to humans during the bite of the female anopheles mosquito. The growing resistance of the parasites to treatment with known anti-malarial agents such as chloroquine is of grave concern and is responsible for some of the worst cases of malaria in the tropical world.² The spread of resistance of the mosquito vector to currently available insecticides and the limited success of potential anti-malarial vaccines contributes to the urgent necessity of finding new chemotherapeutic agents for the treatment of malaria, in particular, agents effective against *P. falciparum*, the strain responsible of the most severe forms of malaria.

The standard test for screening potential drugs for anti-plasmodial activity is a radioactivity-based method that relies upon the incorporation of ³H-hypoxanthine into the DNA of the parasite to measure parasitic replication in red blood cells.³ This method is very sensitive and it can be used to screen a large number of compounds, but requires hazardous radioactive materials that require special facilities and procedures. Alternatives to the ³H-hypoxanthine-based methodology include a labor-intensive and time-consuming microscopic method and several colorimetric assays.^{4–6} Colorimetric methods, however, are based on enzymatic activity rather than parasite replication, and in addition, may be subject to artifacts caused by pigments present in crude plant extracts that are frequently used in drug screening programs.

Traditionally, natural products have been a rich source of anti-plasmodial drugs, including quinine and artemisinin,^{7,8} many of which are derived from biodiversity-rich developing countries. Since the standard anti-plasmodial assay is based on the use of radioactive isotopes, the same developing countries are often not in a position to develop anti-malarial drug discovery programs, limiting access to a large pool of scientific talent and emphasizing the need to develop cost-effective techniques that do not require the use of radioactive isotopes.⁹ The present study proposes a new, straightforward, efficient, and accurate method for the detection of anti-malarial agents based upon the intercalation of the fluoro-

chrome PicoGreen[®] into *Plasmodium* DNA. PicoGreen[®] is an ultrasensitive fluorescent nucleic acid stain for measuring double-stranded DNA (dsDNA) in solution, and it enables the detection of quantities as low as 25 pg/mL of dsDNA with a moderately priced spectrofluorometer using fluorescein excitation and emission wavelengths. Accordingly, the microfluorimetric method described herein is ideally suited for anti-malarial drug discovery programs based in developing nations.

MATERIALS AND METHODS

Cultivation of parasites. Two chloroquine-sensitive (Sierra Leone clone D6 and Tanzania F32) strains and a chloroquine-resistant (Indochina clone W2) strain of *P. falciparum* were used for this study. The D6 clone was provided by Philip J. Rosenthal (Division of Infectious Diseases, University of California, San Francisco, CA). The W2 clone was provided by Dennis Kyle (Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD). The F32 strain was provided by Eric DeHaro (Institut de Recherche pour le Développement Group, Instituto de Investigaciones Fármaco Bioquímicas, Universidad Mayor de San Andres, La Paz, Bolivia).

The three strains were maintained *in vitro* by a modification of the method of Trager and Jensen.¹⁰ The culture media consisted of standard RPMI 1640 (Gibco-BRL Laboratories, Gaithersburg, MD) supplemented with 10% heat-inactivated human type O+ serum (Valley Biomedical, Inc., Winchester, VA), 25 mM NaHCO₃, 2 mM glutamine, and 25 HEPES (Sigma, St. Louis, MO). Cultures were maintained in type O+ human red blood cell suspensions obtained from healthy local donors and prepared in citrate-phosphate-dextrose anticoagulant (Sigma) at a hematocrit of 2%. The parasite density was maintained below 2% parasitemia under an atmosphere of a certified gas mixture containing 5% CO₂, 5% O₂, and 90% N₂ at 37°C. For each experiment, samples of stock cultures were further diluted in culture medium containing sufficient noninfected type O+ human erythrocytes to yield a final hematocrit of 2% and a parasitemia of 1%. All assays were carried out in microtiter plates. For those cases in which assays were synchronized, sorbitol was used.¹¹

Radioactivity-based assay. Incorporation of ³H-hypoxanthine (specific activity = 1.0 mCi/mL; American Radio-

labeled Chemicals, Inc., St. Louis, MO) was used to measure growth of the parasites, as previously described by Desjardines and others.³ Different antimalarial compounds at final concentrations ranging from 1.95 nM to 2 μ M were added in duplicate to flat-bottom, 96-well microtiter plates (Corning Glass Works, Corning, NY) in a final volume of 25 μ L. A 200- μ L volume of the culture parasite was added to each well and the plate was then placed in a humidified airtight chamber (Bellco Glass Inc., Vineland, NJ) that was flushed with the gas mixture described earlier, sealed, and stored in an incubator at 37°C for 24 hours. Each compound was tested on at least two occasions against both chloroquine-sensitive and chloroquine-resistant strains. At the end of the incubation period, 25 μ L of diluted ³H-hypoxanthine (final concentration = 1.5 μ Ci) was added to each well. The plates were then returned to the humidified airtight chamber, flushed again with the gas mixture described earlier, sealed, and incubated at 37°C for an additional 18 hours. The cultures were then harvested with a semi-automated PHD Cell harvester[®] (American Instrument Exchange, Inc., Haverhill, MA) onto fiberglass paper disks, washed with distilled water, and fixed with ethanol. Each disk was placed in glass scintillation vials containing 2 mL of Microscint scintillation cocktail (Microscint-High Efficiency LSC-Cocktail; Perkin Elmer Life and Analytical Science, Boston, MA) for one hour. The vials were then counted in a Packard microplate scintillation beta counter (American Laboratory Trading LLC, Niantic, CT). The mean values for uptake of ³H-hypoxanthine in parasitized control and nonparasitized control erythrocytes were calculated.

Fluorimetric susceptibility test. Synchronized ring form cultures (hematocrit = 2% and parasitemia = 1%) were used to test pure compounds or serial dilutions of plant extracts in 96-well culture plates. Cultures of *P. falciparum* were placed in a humidified, air-sealed container, flushed with the gas mixture described earlier, and incubated at 37°C. Parasites were allowed to grow for a 48-hour incubation period, after which a 150- μ L aliquot of culture was transferred to a new 96-well flat bottom plate. Fifty microliters of the fluorochrome mixture, which consists of PicoGreen[®] (Molecular Probes, Inc., Eugene, OR), 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE buffer), and 2% Triton X-100 diluted with double-distilled, DNase-free water, was then added to liberate and label the parasitic DNA. The plates were then incubated for 5–30 minutes in the dark. The fluorescence signal, measured as relative fluorescence units (RFU) was quantitated with a fluorescence microplate reader (FL_x 800; Bio-Tek Instruments, Inc., Winooski, VT) at 485/20 nm excitation and 528/20 nm emission. Simultaneously, the RFU from positive and negative control samples were obtained, stored, and analyzed.

Preparation of crude plant extracts and microtitration plates. Plant samples were prepared according to standard protocols.¹² Lyophilized crude extracts were provided in individual vials of 3 mg (dry weight) and stored at –20°C until ready for testing. Crude extracts and partially-purified fractions were dissolved in dimethylsulfoxide (DMSO) (Research Organics, Cleveland, OH) at a stock concentration of 50 mg/mL. Known antimalarial compounds were dissolved in distilled water or ethanol according to published methods.^{13,14} Samples were tested in 96-well plates in duplicate at final concentrations of 50, 10, and 2 μ g/mL and re-evaluated at

higher or lower concentrations when necessary. The final dilution contained less than 0.1 DMSO, which had no measurable effect on parasite survival in this system. DMSO at a final concentration of 0.1% in RPMI 1640 culture media was used as negative control, and represented 100% parasite viability. The positive control consisted of chloroquine at concentrations of 1.0, 0.1, and 0.01 μ g/mL, and provided a measure of the susceptibility of the parasite to known antimalarial drugs. To measure the effect of each plant extract alone on the fluorescence signal, each extract concentration was incubated in the absence of parasites and the signal was subtracted from the value obtained in the presence of drug and parasite.

Data analysis. Data analyses were performed with a pre-programmed calculus sheet on Microsoft (Redmond, WA) Excel[®] 2000 that processes the relative fluorescence units exported through the KC junior software from the microplate fluorimeter. The calculus sheet consists of 1) a formula that calculated the mean of the two replicates per sample condition, 2) subtraction of the respective color background of each dilution of the plant extract, 3) conversion of the mean RFU value to percentage of the response, taking as 100% the mean of the negative control, and 4) conversion of the percentage to the 50% inhibitory concentration (IC₅₀) by log-regression. To adjust for the potential contribution of the hemoglobin pigment from erythrocytes and the possible fluorescence from the intrinsic pigments present in some plant extracts, control wells were used that consisted of noninfected erythrocytes alone, and samples of diluted drugs or extracts with noninfected erythrocytes. The inhibitory concentration (IC₅₀) was defined as the drug concentration that results in 50% of the net fluorescence compared with nontreated control cultures.

RESULTS

Relationship between parasite number and fluorescence. Preliminary experiments demonstrated that serial dilutions of normal uninfected red blood cells did not emit significant amount of fluorescence when incubated in the presence of PicoGreen[®], indicating that DNA from contaminating white blood cells and the hemoglobin pigment from erythrocytes does not interfere with the detection of *Plasmodium* DNA. In addition, serial dilutions of crude plant extracts, either alone or mixed with uninfected erythrocytes, also failed to produce significant fluorescence, suggesting that any pigments associated with crude plant extracts do not interfere with the fluorescence signal associated with *Plasmodium* DNA.

To test the sensitivity of the fluorimetric method as a means of detecting *Plasmodium* DNA in infected erythrocytes, we compared the percentage of infected erythrocytes as determined by microscopic counting with results obtained from the fluorimetric technique. We used serial double dilutions of infected erythrocyte cultures to prepare Giemsa-stained thin blood smears and the percentage of parasitemia was then evaluated by light microscopy. Aliquots of the same or parallel cultures were mixed in a 96-well plate with an equal volume of PicoGreen[®] cocktail and the amount of fluorescence was quantified as described in the Materials and Methods. As shown in Figure 1, there is a direct relationship between the percentage of infected red blood cells and the fluo-

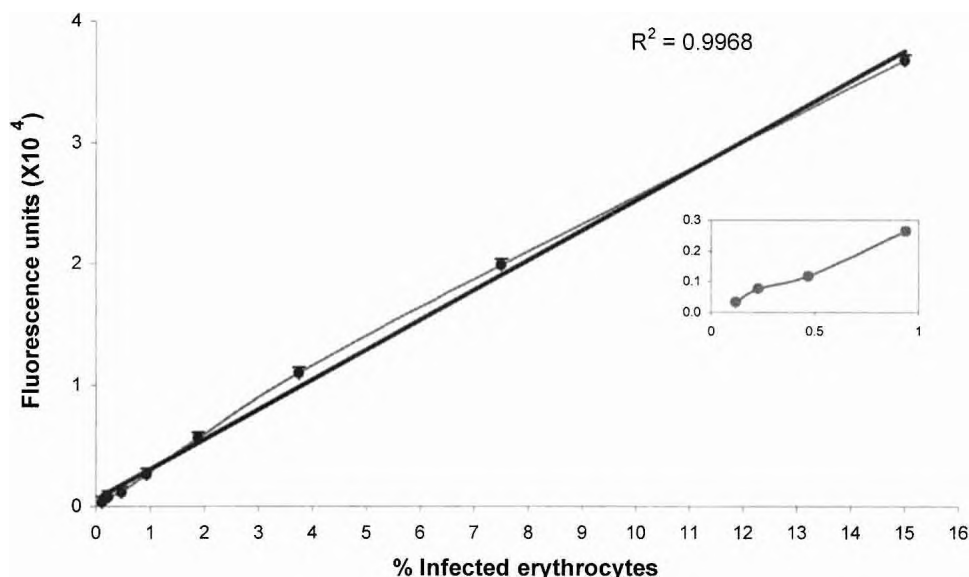


FIGURE 1. Comparison of the percentage of *Plasmodium falciparum*-infected erythrocytes determined by microscopic counting with fluorescence intensity obtained from the microfluorimetric technique. A serial two-fold dilution of a synchronized infected culture (15.0% ring stage) with noninfected erythrocytes was used. Bars indicate the standard deviation of the mean for four independently processed samples. The inset shows the relationship below 1% of parasitemia.

rescence signal between 0.1% and 15% of ring stage infected erythrocytes ($r = 0.99$).

Time course for the assessment of parasitemia. Time course experiments were then performed in which cultures of *P. falciparum*-infected erythrocytes were initiated at a parasitemia of 0.5% and the number of parasites was determined at different time intervals by both microscopic counting and the microfluorimetric technique. Figure 2 shows that both methods of detection are equally effective in detecting the

presence of infected erythrocytes. No differences were observed when nonsynchronized or D-sorbitol-synchronized *Plasmodium* cultures were used, nor were differences observed when chloroquine-sensitive (F32 and D6) or chloroquine-resistant (W2) strains were tested. Based upon these experiments, a time point of 48 hours was chosen for the evaluation of potential anti-plasmodial compounds.

Determination of IC₅₀ values of known antimalarial drugs. The microfluorimetric method was used to determine

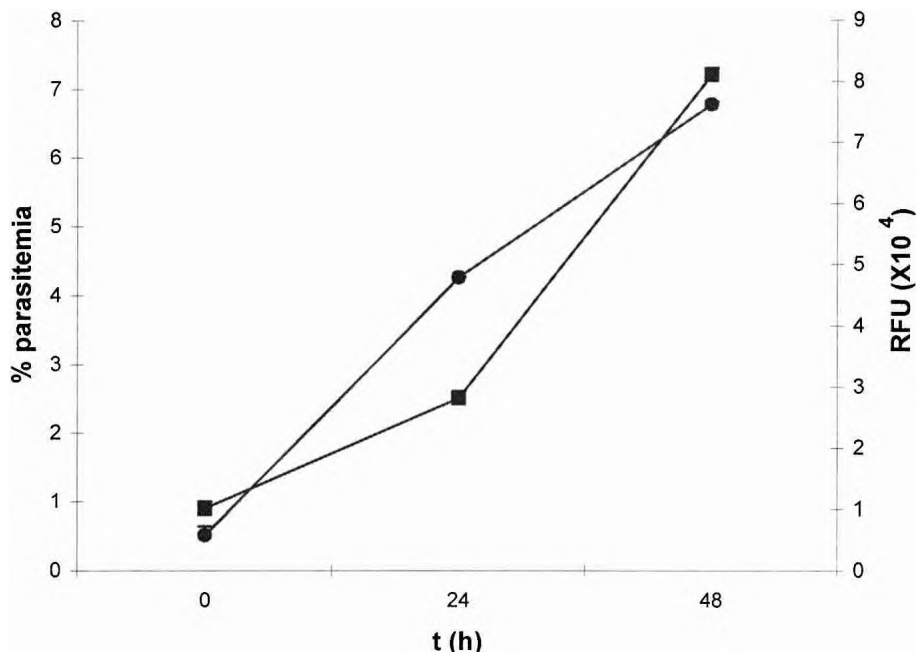


FIGURE 2. Time course experiments with *Plasmodium falciparum*-infected erythrocytes by microscopic counting and the microfluorimetric techniques. Parallel cultures of synchronized parasites were initiated at a parasitemia of 0.5% and analyzed at 24 and 48 hours (h). Bars indicate the standard deviation of the mean for two independently processed samples. RFU = relative fluorescence units.

the effect of known antimalarial drugs on the growth of *P. falciparum* by testing the effect of chloroquine and mefloquine on the growth on the F32 strain, a chloroquine-susceptible parasite. From dose-response experiments, an IC_{50} of 31 ± 0.7 nM (mean \pm SD) for chloroquine was determined using the microfluorimetric method, which is comparable to the previously reported value of 29 ± 9 nM determined by 3H -hypoxanthine incorporation.¹⁵ The IC_{50} for mefloquine was 15 ± 3.7 nM, which is comparable to the value of 9.2 ± 4.2 nM that was determined with the radioactivity-based method.¹⁴ The dose response curves obtained with the radioactivity-based and microfluorimetric methods for measuring the effect of chloroquine on the growth of the chloroquine-resistant W2 clone are shown in Figure 3. We did not observe any significant difference in the IC_{50} values determined by either method, yielding IC_{50} values of 86.5 ± 9 and 88.7 ± 0.72 nM for the radioactivity-based and microfluorimetric methods, respectively. The IC_{50} values determined for chloroquine in these experiments are comparable to the published value of 128 ± 73 nM for the chloroquine-resistant strains.^{5,15}

Drug discovery. Natural products from plants have been a rich source of anti-parasitic compounds.^{7,8} Therefore, we evaluated the ability of the microfluorimetric method to detect plant extracts with anti-plasmodial activity and to assess

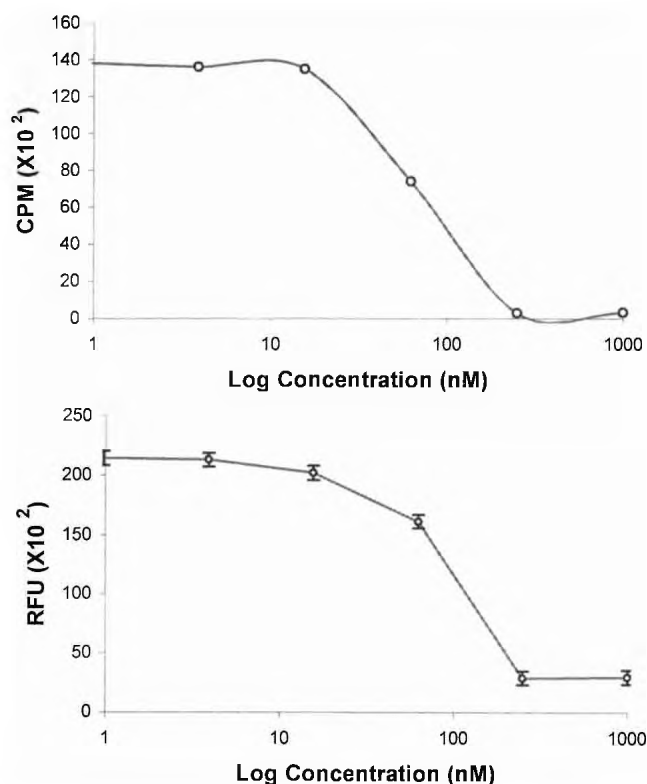


FIGURE 3. Determination of the 50% inhibitory concentration (IC_{50}) values for chloroquine by the incorporation of 3H -hypoxanthine (top) and the microfluorimetric technique (bottom). Cultures of *Plasmodium falciparum* W2 strain-infected erythrocytes were initiated at a parasitemia of 0.5%, incubated with different concentrations of chloroquine, and the number of parasites was determined at 48 hours. IC_{50} values of 88.7 and 86.5 $\mu g/mL$ were determined for the microfluorimetric and radioactivity-based assays, respectively. Bars indicate the standard deviation from the mean for four independently processed samples. CPM = counts per minute; RFU = relative fluorescence units.

its utility as a systematic and efficient means of screening large numbers of crude extracts. We considered as active those plant extracts with IC_{50} values < 50 $\mu g/mL$. Table 1 shows that there was a perfect correlation between the radioactivity-based, microscopic, and microfluorimetric techniques with respect to their ability to detect plant extracts with anti-plasmodial activity (seven of seven extracts tested with the three assays and two of two extracts tested with the fluorimetric and radioactivity methods). While the IC_{50} levels of crude extracts measured by the radioactivity-based and microscopic methods tend to be lower than those values measured by the microfluorimetric assay, no differences were observed in IC_{50} values when pure compounds were evaluated (Figure 3). We carried out the complementary experiment in which plants shown to be inactive by the radioactivity-based method were tested in the microfluorimetric assay. In every case (five of five), plants that were inactive in the radioactivity-based assay were also inactive in the microfluorimetric method, an observation relevant to the use of the latter method for drug discovery (Table 1).

The microfluorimetric assay was used to guide the purification of a compound with anti-*Plasmodium* activity from the plant *Coccoloba parimensis*. Initial screening of a crude extract of leaves of *C. parimensis* demonstrated significant anti-plasmodial activity ($IC_{50} = 6$ – 12 $\mu g/mL$). The extract was subjected to liquid-liquid partition with hexane, ethyl acetate, methanol and water, a technique used to separate the chemical constituents on the basis of their relative polarity¹² and the fractions were tested for anti-plasmodial activity. Purification of the sample resultant from the ethyl acetate fraction ($IC_{50} = 10$ $\mu g/mL$) led to the isolation of the methyl ester of gallic acid that showed IC_{50} values < 2 $\mu g/mL$.¹⁶

DISCUSSION

The microfluorimetric method for detecting anti-plasmodial compounds described herein has several advantages over the traditional assay that monitors the incorporation of 3H -hypoxanthine by the parasite.³ The radioactivity-based method requires the use of an expensive, hazardous radioactive compound, costly liquid β -scintillation counter

TABLE 1

Comparison of IC_{50} values for crude plant extracts by uptake of [3H]-hypoxanthine, microscopic counting of Giemsa-stained thin blood smears, and the microfluorimetric technique*

Plant extracts	Fluorometric	Radioactivity	Microscopic
<i>Pogonopus speciosus</i>	5	0.2	0.02
<i>Coccoloba parimensis</i>	3	0.1	0.5
<i>Quassia amara</i>	6.5	0.8	0.6
<i>Marmaroxylon dinizii</i>	9	0.9	0.6
<i>Trattinnickia aspera</i>	7	1	4
<i>Simarouba amara</i>	8	1	2
<i>Syzygium jambos</i>	0.9	3	3
<i>Hiraea reclinata</i>	3	8	ND
<i>Nympheia ampla</i>	18	8	ND
<i>Solanum lancefolium</i>	> 50	> 50	ND
<i>Platypodium elegans</i>	> 50	> 50	ND
<i>Dolicarpus multiflorus</i>	> 50	> 50	ND
<i>Cydista heterophylla</i>	> 50	> 50	ND
<i>Carapa guianensis</i>	> 50	> 50	ND

* Values are in micrograms/milliliter.

IC_{50} = 50% inhibitory concentration; ND = not done.

equipment, and special local regulations for the introduction, management, and disposal of radioactive waste. An impediment for the development of drug discovery programs in developing countries is the lack of accessible and appropriate technology that would permit the efficient testing of biologic materials for anti-plasmodial activity. Although several non-radioactivity-based methods have been developed over the years, they are cumbersome, multistep procedures.^{4,5}

The method described herein is based upon the detection of *Plasmodium* DNA in short-term cultures using a 96-well format, allowing the efficient and quantitative measurements of anti-plasmodial activity in a large number of samples. The method uses PicoGreen[®], an ultrasensitive fluorophore that intercalates into the double-stranded DNA of *Plasmodium* in solution, enabling the detection of as little as 25 pg/ml of dsDNA, a 400-fold increase in sensitivity compared with the DNA intercalator Hoechst 33258 (Polysciences, Inc., Warrington, PA).

The PicoGreen[®] method is straightforward and rapid. The parasites are first incubated with the test drug for 48 hours, followed by addition of PicoGreen[®], followed by a 5–30-minute incubation period prior to the measurement of fluorescence. The PicoGreen[®] assay protocol presented herein is simpler than that for Hoechst 33258 since there is no requirement to remove potentially interfering compounds such as hemoglobin and hemozoin, nor is there a chloroform extraction step to prevent quenching of fluorescence.¹⁷ The replication of the parasite is directly proportional to the amount of fluorescence, with a linear relationship between parasitemias of 0.1% and 15%. We have used synchronized and non-synchronized parasites, and observed no significant differences. In addition, the samples can be stored at –20°C and read at a more convenient time without a significant change in the fluorescence signal. Significantly, if a fluorescence microplate reader is not available, determination of parasite growth may be achieved with a less-expensive minifluorimeter (Mini-fluorimeter TKO 100; Hoefer Scientific Instruments, San Francisco, CA).

We compared the microfluorimetric methodology with the conventional radioactivity-based assay by using both methods to test crude plant extracts for anti-plasmodial activity. We found that for all of the extracts tested, both methods yielded identical results. We do not have an explanation for the small differences between the calculated IC₅₀ values of crude plant extracts as determined by the two methods. One possible explanation is the presence of low levels of interfering substances in the extracts. Alternatively, the persistence of *Plasmodium*-derived DNA related to the initial parasite inoculum may be responsible. However, no significant difference in IC₅₀ values were observed between the two methods when pure compounds (chloroquine and mefloquine) were tested, supporting the utility the PicoGreen[®] assay for quantifying anti-plasmodial activity. The microfluorimetric method described herein has been used successfully to guide the purification of compounds with anti-plasmodial activity from crude plant extracts. It is hoped that the development of an effective and straightforward method for measuring anti-plasmodial activity that does not use radioactive isotopes will stimulate anti-malarial drug discovery programs in a number of countries, in particular, those most affected by this deadly disease.

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