Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 880–886

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Screening medicinal plants for the detection of novel antimalarial products applying the inhibition of β-hematin formation

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ARTICLE INFO

Article history:
Received 22 March 2011
Received in revised form 27 June 2011
Accepted 28 June 2011
Available online 5 July 2011

Keywords:
β-Hematin assay
Screening
Plant extracts
Targeted isolation
Malaria

ABSTRACT

The identification of novel scaffolds for the development of effective and safe treatments to fight malaria is urgently needed. One of the main opportunities is the discovery of new molecules from natural origin. A simple, robust and cost-effective colorimetric assay based on the inhibition of β-hematin has been adapted to routinely screen plant extracts with the ultimate goal to identify novel antimalarial ingredients. The development of this assay has included a careful optimization of all critical experimental parameters. The β-hematin assay can be completed in less than one working day, requiring a 96-well UV–vis plate reader and low-cost commercially available reagents using a standard operating protocol. It can be used on its own or in combination with the well-known *Plasmodium* growth inhibition assay and has the obvious merit to be informative at the early stage of drug discovery regarding the mechanism of action of the actives. A total of 40 diverse natural products and 219 plants extracts were tested. Good correlations in respect with specificity (pure compounds 85%, extracts 93%) and positive predictive value (pure compounds 72%, extracts 50%) were obtained in comparison with *Plasmodium* growth inhibition assay that was used as the reference assay.

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1. Introduction

Malaria is a life-threatening disease caused by protozoa of the *Plasmodium* genus that are transmitted to humans through *Anopheles* mosquito bites. There were 243 million cases of malaria in 2008 that have resulted in an estimate 863,000 deaths, mostly among African children [1]. The burden of this major health issue in endemic countries was fuelled in the past years by the emergence of parasite resistance to chloroquine, mefloquine and sulfadoxine-pyrimethamine that looks likely to extend to artemisinin combined therapies in the future [2–4]. First reports of resistance to artemisinin-containing drugs (artesunate) have been reported along the Thai-Cambodian border and shown to be due to a significant reduction of *in vivo* susceptibility to artesunate [5]. Artesunate resistance was indeed characterized by a markedly prolonged time for parasite clearance that could not be explained by pharmacokinetic or other host factors [5]. Resistance has certainly rapidly emerged as a consequence of the widespread use of artemisinin monotherapy in the region [6].

From a biological standpoint, the intraerythrocytic stage appears to be a vital part of the *Plasmodium* life cycle in the human body. It involves digestion of hemoglobin which provides amino acids and energy both essential for the development and the proliferation of the parasite [7]. Among the possible routes sequestration, via biocrystallization of heme into hemozoin appears to be an important mechanism of the heme detoxification [8,9]. The hemozoin formation pathway has been widely studied from both chemical and mechanistic points of view [10–14]. From a drug target perspective this pathway has been validated for several antimalarial molecules including the 4-aminoquinolines (quinine, mefloquine, amodiaquine and chloroquine) and is therefore considered a suitable target for drug discovery programs [15–17]. Several assays targeting the inhibition of the hemozoin (or β-hematin) formation have been developed that have led to the development of screening tools based on different spectrometric methods such as radioisotopic [18], FT-IR [19] and UV–vis techniques [20–26]. Some of these assays using incorporation of ^{14}C-hemin [27] or spectrophotometric quantification of β-hematin [28] were subsequently adapted to the high throughput screening of selected libraries leading to the discovery of several molecules inhibiting the hemozoin (β-hematin) formation. The screening of more than 100,000 non-quinoline compounds by Kurosawa et al. in collaboration with Roche led to the identification of 8 new classes of

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doi:10.1016/j.jpba.2011.06.026

β-hematin inhibitors [27]. The use of radiolabelled chemicals has been limited and has not become part of routine testing due to the cost and length of time required to carry out the assay. Alternative screening assays requiring specific material isolated from *Plasmodium* sp. such as specific fractions or lysates that simulate the *in vitro* formation of hemozoin [21,22] also have their limitations as they require specific and complicated technical skills and equipment to handle *Plasmodium* parasites. As part of the early assay development endeavors, Egan et al. demonstrated *in vitro* the synthesis of β-hematin to occur spontaneously in the presence of an acetate solution under optimized experimental conditions related to temperature and pH [24]. This biosynthetic pathway used for the hemozoin (β-hematin) formation was previously shown to be inhibited by antimalarial drugs from the 4-aminoquinoline family [19,29,31,32]. These observations have since been used to develop a simple and rapid colorimetric (Phi-*b* assay) based on differential solubilisation and coordination of Fe(III)PPIX by aqueous pyridine (Py–Fe(III)PPIX) [30]. The output of this assay is based on the colorimetric detection at a defined wavelength that can be achieved with a standard UV/vis spectrophotometer. In the presence of an inhibitor, the synthesis of β-hematin slows down or even stops [29] and the addition of a buffered pyridine solution in HEPES allows for the selective distinguishing of the synthetic product from hematin. The complex Py–Fe(III)PPIX displays a red coloration and is detectable by spectroscopy UV–vis with a maximum of absorption of 404 nm. The affinity of pyridine to form a complex with the central iron of a porphyrine forms the basis of this technique, which used for the quantification of heme [30]. So far this user-friendly assay has only been utilized for the screening pure compounds, notably the 4-aminoquinolines. More recently, Rush et al. have adapted the Phi-β assay to a 384-well plate format and screened 16,000 small molecules [33]. Carter et al. incorporated lipophilic mediated assays to discover β-hematin inhibitors from natural product extracts [34]. The aim of our work is to adapt and evaluate the potential of the assay developed by Ncokazi and Egan to screen natural products from plant families including complex mixtures such as extracts and fractions. Such an assay would provide a simple, cost-effective, rapid and reliable screening tool for laboratories active in the discovery of antimalarial products of natural origin.

2. Materials and methods

2.1. β-Hematin assay

Hemin from bovine, quinine sulfate, chloroquine diphosphate, and tetrahydrofuran (THF) were purchased from Sigma–Aldrich. Sodium acetate (NaOAc) and HEPES were from Fluka. Pyridine 99%, glacial acetic acid glacial (CH_3COOH) and dimethylsulfoxide (DMSO; 99.7% purity), were obtained from Acros Organics. Hydrochloric acid (HCl) 1 M and sodium hydroxide (NaOH) 0.1 M Titrisol® quality were from Merck. Methanol (MeOH) from Chromanorm was analytical grade. Flat-bottomed 96-well plates of 300 μl and round-bottomed 96-well plates of 2 ml were obtained from Life Technologies-NUNC.

2.2. Sample preparation

The routine preparation of plant extracts used in testing was performed at room temperature with maceration with dichloromethane (CH_2Cl_2, 3 consecutive extractions over 24 h) followed by extraction using MeOH (3 consecutive extractions over 24 h). The use of CH_2Cl_2 was preliminarily used in order to remove more lipophilic compounds, which may hinder the solubilisation of the samples to be tested in MeOH. During the initial work it was shown that a direct extraction in MeOH posed solubilisation issues and it was thus decided to switch to a more rapid extraction protocol requiring only 15 min sonication (L. Haldi Fils) of plant material in MeOH. A total of 12 plants were processed using this second extraction method. The solvent was removed using evaporation under partial vacuum conditions (Büchi Rotavapor) at 40 °C. In both procedures 15 g of powdered plant material were dissolved in 150 ml of solvent.

2.3. SPE purification of plant extracts

A purification method using solid phase extraction (SPE) cartridge filled with 1000 mg of polyamide phase (Macherey-Nagel) was used to remove any interfering material. A total of 150 mg of dry extract were deposited on the cartridge preliminarily conditioned with 6 ml of MeOH and 6 ml of MeOH/H_2O 5:95. A first elution (I) with 6 ml of MeOH/H_2O (5:95) was followed by a second one with 12 ml of MeOH/H_2O 90:10 (II) before a final wash with 6 ml of pure THF (III).

2.4. Optimized protocol of the β-hematin assay

A variety of solutions were prepared for the assay: 1 M HCl; 0.1 M NaOH; 20 mM HEPES; 15% pyridine in HEPES 20 mM (pH 7.5). A saturated acetate solution pH 5.0 was obtained with 18 g of NaOAc, 24 ml of CH_3COOH and 10 ml of water. Fresh hematin (Fe(III)PPIX) solution (1.05 mM) was prepared daily with 6.8 mg of bovine hemin adjusted to 10.00 ml with NaOH 0.1 M. Stock solutions of pure compounds, extracts and fractions were dissolved in HCl 0.1 M/MeOH/DMSO (5:3:2). Natural compounds tested as pure compounds had an average purity between 85 and 99%. They were specially purchased for the present study from various chemical suppliers. The qualitative determination of the β-hematin synthesis inhibition was tested at one concentration only; a 50 mM solution was used for pure compounds, whilst plant extracts and fractions were tested at 25 mg/ml and 15 mg/ml, respectively. The quantitative determination of the β-hematin synthesis inhibition was performed by testing pure compounds from 0 to 50 mM and IC_{50} was calculated by analyzing the dose-response data using GraphPad Prism® (4.0).

Assays were carried out in U-shaped 96-well plates (2 ml) under the following defined procedure: 10 μl of pure compound or alternatively of the extract was mixed with 100 μl of hematin solution and 10 μl of HCl 1 M in triplicate. Plates were shaken at 900 rpm for 10 min, 60 μl of saturated acetate solution pre-warmed at 60 °C, was added to the wells. Final tested concentrations in wells were 2.7 mM for pure compounds, 1.38 mg/ml for fractions and 0.83 mg/ml for extracts, respectively. After incubation at 60 °C during 90 min (Salvis Thermocenter), 750 μl of pyridine solution was mixed. Plates were then shaken again at 900 rpm for 10 min and allowed to settle for 15 min. An aliquot of 100 μl was transferred to a non-sterilized flat-bottomed 96 well-plate (300 μl). The absorbance was read at λ = 405 nm ($A_{Analysis}$) with an EL-808 microplate reader (Bio-Tek Instruments, Inc.).

2.5. Determination of the inhibition of β-hematin

The inhibition of the β-hematin synthesis was determined according to the process described below. For each sample there was a control analysis ($A_{Analysis;Blank}$), which differed from the sample submitted to the analysis ($A_{Analysis}$) by the addition of 750 μl of HEPES 20 mM instead of pyridine, after incubation and was prepared in triplicate. Absorbance of the complex due to the remaining hematin in wells was calculated using the following formula:

$$\Delta A_{Analysis} = A_{Analysis} - A_{Analysis;Blank}$$

For each tested sample, a blank control ($A_{CLT;Blank}$) as well as its blank ($A_{CLTBlank;Blank}$) were prepared in triplicate under the same conditions as described above, but in the absence of hematin. A volume of 750 μl HEPES 20 mM (instead of pyridine) was added to $A_{CLTBlank;Blank}$.

The residual absorbance of the sample independent from the inhibition of the β-hematin complex was calculated using the following formula:

$$\Delta A_{CLT;Blank} = A_{CLTBlank} - A_{CLTBlank;Blank}$$

The resulting inhibition of the β-hematin synthesis induced by the analyzed sample was calculated accordingly:

$$I_{Analysis} = \Delta A_{Analysis} - \Delta A_{CLT;Blank}$$

If $I_{Analysis}$ has a positive value, the assay is considered as positive (active sample) whereas a negative value indicated a negative result.

In order to remove of borderline results and therefore identify true inhibitors of the reaction in the case of the measurement of pure compounds, a chloroquine control and its blank were tested at 25 mM. The threshold absorbance value ($I_{Threshold;pure}$) was arbitrarily defined as the absorbance causing a 25% inhibition of the β-hematin synthesis by chloroquine.

$$I_{CLT;Chloroquine} = \Delta A_{CLT;Chloroquine} - \Delta A_{CLTblk}$$
$$I_{Threshold;pure} = 0.25 x I_{CLT;Chloroquine}$$

A pure compound was ultimately considered as inhibiting the β-hematin synthesis if associated with a positive value according to the following rule:

$$I_{Analysis} - I_{Threshold;pure} > 0$$

2.6. In vitro parasite Plasmodium falciparum growth inhibition assays

The compounds and plant extracts dissolved in DMSO were tested against the intra-erythrocytic form of *P. falciparum* (NF54 strain) with the method based on the [^{3}H]-hypoxanthine incorporation assay [35]. The cytotoxicity was also assessed against host cells (rat skeletal myoblast L-6 cells). The methods were previously described by Ganapaty et al. [36].

3. Results and discussion

3.1. Synthesis of the complex Py–Fe(III)PPIX

The pyridine–hemochrome complex (Py–Fe(III)PPIX) synthesis was monitored spectrophotometrically at a range of concentrations from 280 to 500 nm to determine the optimal wavelength that can be used for the colorimetric readout of the assay (Fig. 1). The Soret band of hematin shifted from 389 to 402 nm upon addition of pyridine 5% buffered at pH 7.5 with 20 mM HEPES. The optimal wavelength used for the colorimetric readout of the assay was set at 405 nm that is the nearest wavelength available on the multiplate reader. As the addition of different portions of pyridine may influence the formation of the complex, it was important to verify this effect by checking the linearity of a score plot where the Lambert-Beer law is valid (Fig. 2). A mixture containing 100 μl of hematin solution, 100 μM in NaOH 1 M, 10 μl HCl 1 M, 60 μl acetate solution followed by 750 μl of solutions with an increasing concentration of pyridine buffered at 7.5 pH together with HEPES 200 mM, was prepared. The red-colored complex Py–Fe(III)PPIX formed between hematin and pyridine was analyzed by spectrophotometry. The Soret band shift visible from 389 to 402 nm, indicated the formation of the complex when pyridine is added to a solution of hematin.

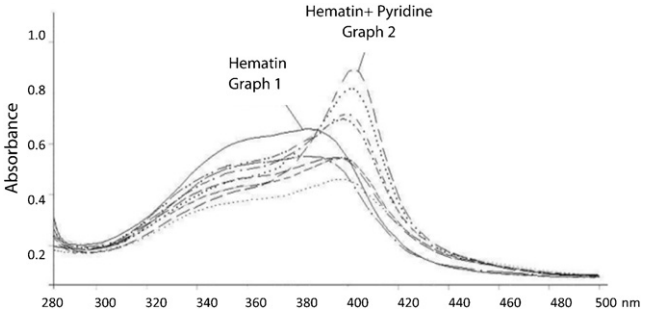

Fig. 1. Determination of the optimum wavelength at the highest peak of absorbance of the complex hematin–pyridine monitored at different concentrations. Graph 1: visible spectrum of 0.15 mM hemin in NaOH 0.1 M at 25 °C before the addition of pyridine 5% in the buffer. Graph 2: the same visible spectrum after the addition of pyridine.

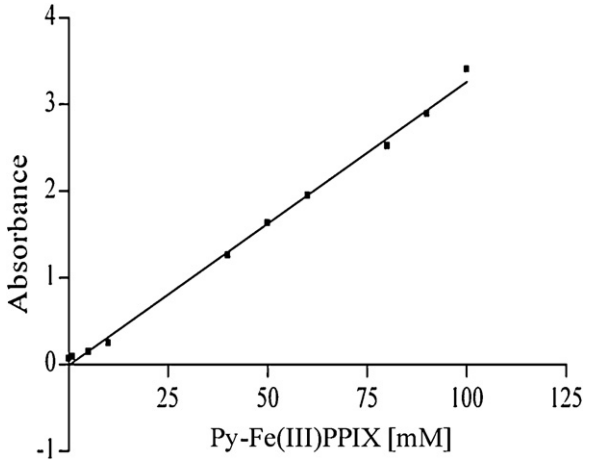

Fig. 2. Absorption at 405 nm of the Py–Fe(III)PPIX complex. Addition of different portions of pyridine.

3.2. β-Hematin synthesis

The protocol for the synthesis of β-hematin was modified from published work by Ncokazi and Egan [30] to adapt it to the screening of plant extracts. Essentially the reaction time and the incubation temperature were identified as the critical parameters and had to be maintained homogeneously across the plates during the transfer of the assay into 96-well plates. A pyridine solution (10%) was added over a period of 0 to 120 min and placed in an oven heated at 60 °C. The time period needed for the formation of the selective hemochrome complex (Py–Fe(III)PPIX) was an effective indicator to follow the progress of β-hematin (Fig. 3). From these results, it was realized that the synthesis is complete after 75 min therefore the incubation time was set at 90 min to ensure

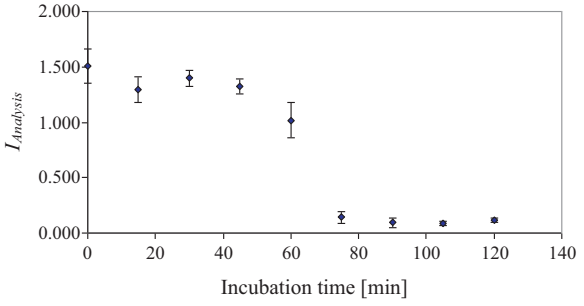

Fig. 3. Optimization of the incubation time for the synthesis of β-hematin. $I_{Analysis} = \Delta A_{Analysis} - \Delta A_{CLT;Blank}$.

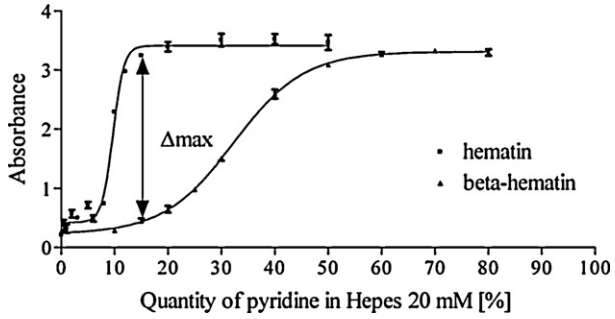

Fig. 4. Selectivity of pyridine towards hematin and β-hematin.

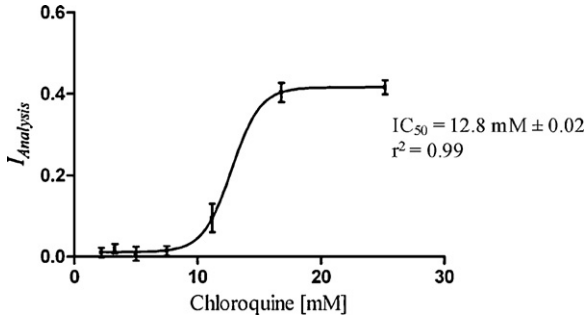

Fig. 6. Quantitative test (0–25 mM) of chloroquine in the β-hematin assay. $I_{Analysis} = \Delta A_{Analysis} - \Delta A_{CLT;Blank}$.

the reaction is followed to completion. The final synthesized product was checked by FT-IR. The two bands relating to the presence of carboxylate groups coordinated to the iron center of a ferriporphyrin were recorded at 1663 cm^{-1} and 1209 cm^{-1}, certifying the structure of β-hematin [37].

The selectivity of pyridine for hematin versus β-hematin was controlled by keeping the pH under 7.5 which is reported to give the best selectivity for hematin [8]. Based on these observations, various concentrations of pyridine in the HEPES buffer solution were tested. Hematin and β-hematin solutions were, respectively, mixed with solutions of pyridine diluted in varying portions with the buffer (HEPES 20 mM). The generation of the complex with Fe(III)PPIX was achieved when an absorbance was detected at λ = 405 nm. The greatest difference in absorbance (Δ_{max}) indicating the best selectivity towards hematin, was obtained with a percentage of pyridine 15%. This concentration of pyridine was therefore selected to run the β-hematin assay (Fig. 4).

3.3. Quantitative β-hematin assay on pure reference drugs

Chloroquine and quinine are known to act as inhibitors of the heme crystallization pathway. They were quantitatively tested on the β-hematin assay at concentrations from 0 to 25 mM. $I_{Analysis}$ values versus their concentration were plotted and resulted in a sigmoid curve, specific to the inhibition of β-hematin synthesis (Figs. 5 and 6). The IC_{50} were determined by fitting the data to a sigmoid dose response curve using GraphPad Prism®. Statistical treatment gave satisfactory results with $r^2 \geq 0.99$ and IC_{50} of 8.6 mM for quinine and 12.8 mM for chloroquine. They were comparable to the IC_{50} values obtained by Egan et al. [39].

3.4. Screening of pure compounds

A set of 40 pure compounds representing the most frequently encountered chemical classes of secondary metabolites in plants were qualitatively tested in the assay at a fixed concentration.

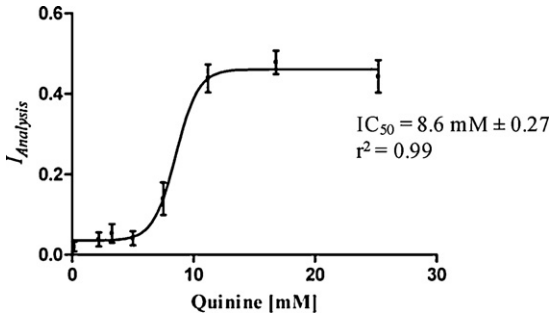

Fig. 5. Quantitative test (0–25 mM) of quinine on the β-hematin assay. $I_{Analysis} = \Delta A_{Analysis} - \Delta A_{CLT;Blank}$.

After addition of the reagents, the final concentration was 2.7 mM. The β-hematin assay is a chemical assay reproducing in a test tube the crystallization of heme into β-hematin. The inhibition of this reaction obeys stoichiometric laws and therefore requires sufficient amounts of reagents (by weight) to lead to quantitative and reproducible results. The use of controls allowed to get rid of the residual interfering absorbance caused by the non-converted hematin as well as by the matrix of the sample. The resulting absorbance was hence exclusively caused by products specifically inhibited the formation of β-hematin under the experimental conditions. To remove borderline results and therefore identified only true inhibitors of the reaction, an additional filter (threshold absorbance value = $I_{Threshold}$) was used. The threshold absorbance value ($I_{Threshold;pure}$) was arbitrarily defined as the absorbance inducing 25% inhibition of the β-hematin synthesis by chloroquine (Fig. 7).

The results (Fig. 8) collected in the β-hematin assay were compared with those obtained on NF54 strains of *P. falciparum* (Table 1). In this later assay, compounds inhibiting less than 50% of the *Plasmodium* growth inhibition at 10 μg/ml were considered as inactive. The β-hematin assay performance was evaluated by calculating the positive predictive value (PPV) in respect with the *Plasmodium* growth inhibition assay used as a gold standard. More specifically, the PPV reflects the probability of the β-hematin assay to predict the positive results obtained using the gold standard assay as described in the following equation:

$$\mathrm{PPV} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false positives}}$$

The assay specificity of the β-hematin assay is defined by the negatives results, which are correctly identified in respect with the

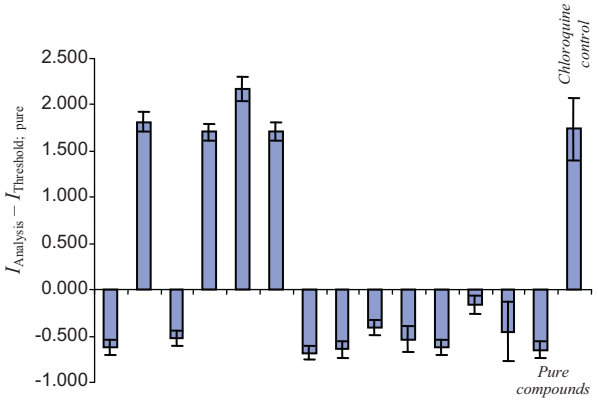

Fig. 7. Example of the screening of pure compounds with chloroquine as control (3 equivalent = 25 mM). Application of the data processing: hits are shown as compounds having a resulting absorbance above the *x*-axis ($I_{Analysis} - I_{Threshold;pure} > 0$).

Table 1
Pure compounds tested in the β-hematin assay and compared with *P. falciparum* whole growth inhibition assay.

Chemical class	Compound trivial name	β-Hematin assay [2.7 mM], $I_{Analysis} - I_{Threshold;pure} > 0$	*In vitro P. falciparum* assay (NF54), IC_{50} [μg/ml]
Cardenolides	Digoxin	–	>10
Coumarins	Umbelliferone	–	>10
Flavanols	Catechine	+	>10
	Epicatechine (–)	+	>10
Flavones	Apigenin	+	5.37
	Luteolin	+	2.45
Flavone glycosides	Diosmin	–	>10
	Vitexin 2″-O-rhamnoside	–	>10
	Apigenin 7-glucoside	–	2.40
Flavonols	Isorhamnetin	–	5.92
	Quercetin	–	2.91
	Morin	+	>10
Flavonol glycosides	Isoquercitrin	+	7.16
	Myricitrin	+	6.59
	Rutin	–	>10
Iridoids	Aucubin	–	>10
Isoquinoline alkaloids	Emetine	–	0.15
Monosaccharides and	Galactose	–	>10
Oligosaccharides	Glucose	–	>10
	Saccharose	–	>10
Monoterpenoids	Thymol	–	>10
Naphtoquinones	Juglon	–	4.87
	Lapachol	–	8.11
	Plumbagin	–	0.417
Organic acids	Malic acid	–	>10
Phenols	Ellagic acid	–	0.17
	Chlorogenic acid	–	>10
	Arbutin	–	>10
Phenylpropanoids	Curcumin	–	1.53
Phytosterols	Sitosterol	–	7.72
Quinoline alkaloids	Cinchonidine	+	0.0063
	Quinidine sulfate	+	0.0047
	Quinine	+	0.0010
Quinones	Anthrone	–	0.546
	Rhein	–	>10
	Emodin	–	>10
Tannins	Hamamelitannin	+	0.63
	Tannic acid	–	0.411
Tropane alkaloids	Atropine	–	>10
	Scopolamine	–	>10

results of the *Plasmodium* growth inhibition assay. The assay specificity is calculated as:

$$\text{Specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}}$$

The results summarized in Table 2 showed that the β-hematin assay correlated with the *P. falciparum growth* inhibition assay with PPV of 72% and a specificity of 85%.

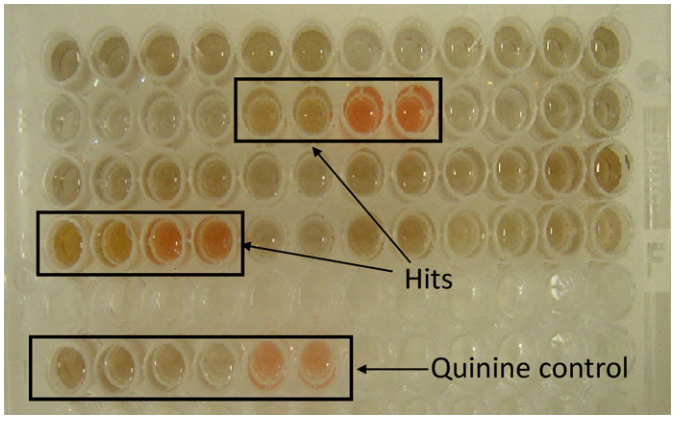

Fig. 8. Screening of samples using the β-hematin assay including example of hits. A quinine control is visible in orange on the plate.

3.5. Screening on plant extracts

A total of 219 plant extracts from our collection, representing 64 botanical families were screened during the investigation at a final concentration of 1.38 mg/ml. The selection of this material was based on different criteria, plants belonging to botanical families/genera known to be used traditionally to treat malaria or/and having been reported for antiplasmodial activities as captured by our lab internal database. A set of 88 plant extracts exhibited a significant inhibition ($I_{Analysis} > 0$) on the synthesis of β-hematin. The activities were compared with activities determined in *P. falciparum* growth inhibition assay. Table 3 presents the correlation between the two assays.

In Table 3, the calculated PPV and specificity were 38% and 89%, respectively. To reduce the number of false positives, a preliminary SPE purification step was evaluated on the same sets of samples. The 88 extracts were filtered on SPE polyamide cartridges and the

Table 2
Pure compounds: correlation between the β-hematin and the *in vitro Plasmodium falciparum* growth inhibition assays.

	In vitro P. falciparum (NF 54 strain)	
	Positive (IC_{50} < 10 μg/ml)	Negative (IC_{50} > 10 μg/ml)
β-Test		
Positive	8 (20%) true positives	3 (8%) false positives
Negative	13 (31%) false negatives	17 (41%) true negatives

Table 3
Plant extracts: correlation between the β-hematin and the *in vitro P. falciparum* growth inhibition assays.

	In vitro P. falciparum on NF 54	
	Positive(IC_{50} < 10 μg/ml)	Negative (IC_{50} > 10 μg/ml)
β-Test		
Positive	5 (6%) true positives	8 (9%) false positives
Negative	6 (7%) false negatives	69 (78%) true negatives

Table 4
IC_{50} correlation values of β-test after SPE step with the *in vitro* antiplasmodial assay.

	In vitro P. falciparum (NF 54 strain)	
	Positive (IC_{50} < 10 μg/ml)	Negative (IC_{50} > 10 μg/ml)
β-Test		
Positive (confirmed by SPE)	5 (6%) true positives	5 (6%) false positives
Negative	6 (7%) false negatives	72 (81%) true negatives

collected fractions were tested in the assays. Table 4 presents a comparison of the results obtained after the clean-up procedure. PPV and specificity reached higher values with 50% and 93%, respectively. The use of a preliminary cleaning step removed a large part of the interfering compounds/frequent hitters encounter in natural mixtures such as tannins; three hits were decidedly ruled out of the hit list based on this technique. From the results presented in Table 4, the PPV was significantly increased as indicated by the deprioritisation of these three false positive samples. The SPE purification has therefore led to a significant improvement of the correlation between the β-hematin assay and the *P. falciparum* growth inhibition assay justifying the cost and labor related to this additional purification step. Relatively higher concentrations of hematin and of tested material (millimolar range) were used to run the β-test in comparison with previous experimental studies performed in the micromolar range by Dorn and coworkers [15,27]. Such high amounts of compounds could potentially lead to some false positive results caused by lack of solubility or precipitation of the tested solutions. Specificities related to the *in vitro* intra-erythrocytic *Plasmodium* testing system such as the presence of biological membranes that may prevent potential β-hematin inhibitors from interacting with the hemozoin pathway and a possible activation and/or accumulation of metabolites in the food vacuole may alternatively explain diverging results between the 2 assays. In the case of true positives, the β-hematin assay has the merit to be informative; the mechanism of action responsible for their antiplasmodial activity can be assessed. A particular example is illustrated by the 4-aminoquinolines. Since those inhibitors of the hemozoin formation pathway do not act on a target, but rather interfere with a physiochemical process, they are unlikely to face drug cross resistance issues related to 4-aminoquinolines, particularly chloroquine. The heme crystallization pathway remains unaltered in resistant parasites as resistance occurs through a membrane transport protein that leads to a significant decrease of the concentration of chloroquine in the food vacuole. Conducting a screening survey based only on the β-hematin assay could however be seen as a limitation for compounds acting through alternative mechanisms that will not be detectable in the β-hematin test. False negative compounds may therefore exert their antiplasmodial activity via others mode of action or may only give positive responses in the chemical assay when higher concentration are used. Physical artifacts due to the relatively poor solubility of the active compound in the aqueous media at such higher concentrations or the precipitation of the hematin protein may also hinder the reaction. Some polyphenolic products such as flavonoids and tannins also responded positively in the β-hematin assay. These compounds have however been reported for their antiplasmodial properties and are known to act through rather unspecific mechanisms of action [38].

This modified version of the β-hematin assay demonstrated to work well in the case of the detection of pure compounds yielding results similar to the ones previously reported for chloroquine and quinine used as reference drugs [30,39]. The activity of plant extracts was also evaluated. A total of 219 plant extracts (64 botanical families) were screened. The results were successfully compared with those obtained in the *P. falciparum* growth inhibition assay used by several laboratories as the golden standard in the antimalarial drug screening field. PPV values of 72% (pure compounds), 38% (extracts without SPE) and 50% (extracts with SPE) and specificity of 85% (pure compounds), 89% (extracts without SPE) and 93% (extracts with SPE) pointed out the good performance of the test in respect to the *P. falciparum* whole cell reference assay. The colorimetric test was able to detect a large portion of samples with potentially interesting antiplasmodial activities. The predictability measured in terms of PPV seems higher when working with pure compounds than extracts or fractions. Although this observation was not unexpected when working with mixtures, it may not be appropriate to draw an early conclusion at this stage between results obtained on pure compounds and extracts due to the a possible bias of selection of the studied material and the limited number of samples tested in those assays.

Overall, the results of the β-hematin assay correlated well with those obtained using the standard *in vitro* antiplasmodial activities based on the inhibition of growth of *P. falciparum (P. falciparum)* measured with the ^{3}[H]-hypoxanthine assay [35,36]. The selection of several known inhibitors on the hemozoin formation pathway from the 4-aminoquinoline class and of additional compounds (flavonoids and tannins) that may have exerted activity via less specific types of inhibition—has probably largely contributed to the large proportion of hits detected in the β-hematin assay that have reconfirmed in the whole cell *Plasmodium* growth inhibition assay leading to a possible misbalance of the percentage of hits active in both assays in regard to the results that could be expected from the screening of a less biased library of compounds. It is more difficult to comment on the extract side as the composition of the investigated material is less known and more complex. Considering the limited number of screened samples and the way those have been selected (several families been overrepresented compared to others) we cannot exclude a bias leading to a higher common hit rate (extracts showing activity in both assays) in comparison to a larger and randomized library of extracts.

The β-hematin assay has the advantage of identifying inhibitors of a well-established and drug validated pathway of the *Plasmodium* parasite, namely the hemozoin formation pathway. Another clear advantage of the β-hematin assay adapted to the screening of natural origin is its simple and rapid protocol. The assay can easily be performed within one working day, avoiding handling of *Plasmodium* cultures and only requiring rather basic laboratory equipment. This assay can therefore be quite smoothly implemented in natural product laboratories, including those located in endemic areas as a suitable tool for antimalarial drug discovery. The β-hematin assay can be used together with an analytical dereplication technique such as LC/UV/MS coupled to a MS database of well-known/ubiquitous compounds that could dramatically speed up the hit triage decision process and allow focus on quality material of high potential for subsequent research activities. As the hemozoin formation pathway is also present in *Schistosoma*, the assay can be used for drug discovery against schistosomiasis though the inhibition of this pathway is not has thoroughly validated in terms of drug discovery as it is in the case of *Plasmodium* [40,41].

Funding

The work was supported by the Swiss National Science Foundation (grant no. 200020-107775/1).

Acknowledgements

The authors are grateful to Medicines for Malaria Venture (MMV), Geneva, Switzerland for the access to the biological assays performed on *P. falciparum*. Special thanks are due to Timothy Egan for his expert recommendations related β-hematin assay made in the frame of this work, as well as to Georgina Bingham and Harparkash Kaur for their critical reading of the manuscript. This paper is dedicated to Prof. Dr. h.c. Gerhard Bringmann on the occasion of his sixtieth birthday.

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