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Beta Hematin Inhibition: Evaluating the Mechanism of Action of Some Selected Antimalarial Plants

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ABSTRACT

There is paucity of information regarding the mechanism of action of medicinal plants with antimalarial activity. Thus, the mechanism of action of twenty antimalarial plants using the β-hematin inhibition assay was evaluated. Beta-hematin synthesis from bovine hemin in the presence of saturated acetate solution at a temperature of 60°C and pH 7.5 was initiated with a fixed concentration of 0.69 mg/mL of chloroquine or methanol extracts of the selected plants. The fifty percent inhibitory concentration (IC_{50}) of the six most active plant extracts/standard drug was determined using linear regression. Two of the plant extracts *A. boonei* and *M. charantia* (IC_{50} = 0.09 ± 0.03 and 0.11 ± 0.02 mg/mL), showed significant activity than chloroquine (0.36 ± 0.16 mg/ml. $P<0.05$) a potent β-hematin inhibitor. The β-haematin colorimetric assay is a reliable assay for determining the mechanism of action of medicinal plants that utilize the pathway.

Keywords: Beta haematin, antimalarial plants, Nigerian ethnomedicine

INTRODUCTION

Malaria is a major public health problem in sub-Sahara Africa with high rate of morbidity and mortality common in children less than 5 years of age and pregnant women [1]. In the last five years, malaria incidence rates (new malaria cases) fell by 37% globally and by 42% in Africa. During this same period, malaria mortality rates fell by 60% globally and by 66% in the African Region[2]. The recent worldwide gain achieved in reducing malaria burden is threatened by emergence and spread of drug resistance to artemisinins the most effective antimalarial available today[3-5]. Thus, there is need to enrich the antimalarial drug discovery pipeline with new effective agents and explore the mechanism of action of promising antimalarial agents at the early stage of drug discovery. Fortunately, several unique

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pathways have been identified such as; inhibition of DNA synthesis, de novo haem biosynthesis, glycolysis and hemozoin formation that can serve as drug targets[6-9]. Inhibition of hemozoin formation was explored in this study. Hemozoin is an insoluble, nontoxic disposal product formed from the digestion of haemoglobin by some blood-feeding parasites. These hematophagous organisms such as *Plasmodium sp., Rhodnius sp. and Schistosoma* sp. digest haemoglobin and release high quantities of free haem, which rapidly oxidizes to toxic Fe(III) hematin and is further sequestered as a nontoxic crystalline hemozoin also known as β-hematin[10-12]. The formation of hemozoin is essential to the survival of these parasites and can be considered as an attractive target for developing drugs for the related diseases. Quinine, and its synthetic homologs chloroquine, mefloquine, and others[13-15] putatively work by blocking conversion of hematin to a non-toxic crystalline hemozoin[16]. Similarly, artemisinin, another antimalarial drug of plant origin has been shown to bind to haem[17-18]. Effort in this report was directed towards the determination of the mechanism of action of twenty medicinal plants selected from Nigerian antimalarial compendium through the β-hematin inhibition assay.

METHODOLOGY

Plants Selection, Collection and Authentication

Twenty plants were selected through an extensive literature search of plants used for the treatment of malaria. Twenty plant species from 16 different families were collected, and identified by a plant taxonomist at Forestry Research Institute of Nigeria (FRIN), Ibadan in March 2013.

Extraction of Plant Materials

Powdered plant material (200 g) were percolated in 70% methanol for 72 h. Methanol extracts were filtered and concentrated under vacuum at 40°C using a rotary evaporator. Thereafter, the marc was further percolated in 70% methanol and processed as described above. The extraction process was repeated 2 times for exhaustive extraction of all plant materials. The yields of the methanol extracts were determined and extracts stored at -20°C until needed for study.

Reagents

Bovine hemin, pyridine anhydrous 99.8%, hydroxyethylpiperazine-N-[2-ethanesulfonic acid] (HEPES), sodium acetate, acetic acid, sodium hydroxide (NaOH) and chloroquine diphosphate were obtained from Sigma Aldrich Co. St. Louis, U.S.A. Hydrogen chloride (HCl), methanol and dimethyl sulfoxide (DMSO).

Qualitative Determination of Inhibition of ß-Haematin Synthesis of Twenty Selected Medicinal Plants

The assay was performed based on the method of Vargas and co-workers[19]. A stock concentration (25 mg/mL) of the plant extract/standard antimalarial drug (chloroquine) was prepared in aliquot of DMSO and subsequently diluted in solution containing 0.1M HCl, methanol and DMSO in the following ratio 5:3:1 to give 12.5 mg/mL working solution. The final concentration after addition of various reagents in the test plate (24-well plate) resulted in 0.69 mg/mL. Briefly, 10 µL of plant extract 1, 2 and chloroquine were dispensed into different wells in column 1 - 6 in duplicates. In this format two extracts and a standard drug (chloroquine) were tested in the 24-well plates. In addition, 10 µL of 1M HCl was added to all plant extracts or chloroquine in the 24-well plates. Into the wells in row A and B (the first two rows) 100 µL of freshly prepared hematin solution was added while wells in row C and D received 100 µL of 0.1M NaOH. The test plate was shaken at 450 rpm for 10 min. This was followed by addition of 60 µL of saturated acetate solution, pre-warmed at 60°C into all the wells. The test plate was further incubated at 60°C for 90 min. Thereafter, 750 µL of 15% pyridine was added to wells in the first and the third rows (row A and C) while 750 µL HEPES (pH 7.5) was added to wells in the second and the fourth rows (row B and D). Furthermore, the test plate was shaken at 450 rpm for 10 min and allowed to settle for 15 min. An aliquot of 100 µL was transferred in duplicates to a new 96-well plate and absorbance read at 405 nm with a spectrophotometer (SPECTRAmax GEMINI XS, Molecular Devices, USA).

Following the initial screening, methanol extract of 6 most active medicinal plants were quantitatively tested on the β-hematin assay at concentrations ranging from 0.04 to 1.38 mg/mL.The plant extract/drug concentrations were plotted against $I_{Analysis}$. The IC_{50} of the plant extract/standard drug was determined using a linear regression in a commercially available statistical package Microcal Origin®.

Data Analysis

For each plant extract/drug tested, (the absorbance, represented as $A_{Analysis}$) there was a control analysis ($A_{Analysis;\ Blank}$), which differed from the plant extract/drug submitted for the analysis ($A_{Analysis}$) by the addition of 750 µL of 20 mM HEPES instead of pyridine, after incubation. In addition, for each plant extract/drug, a blank control ($A_{CLT;\ Blank}$) as well as its blank ($A_{CLTBlank;\ Blank}$) were prepared in the absence of hematin but with 750 µL of 15% pyridine or 750 µL of HEPES.

1. Absorbance of the complex due to the remaining hematin in wells was calculated using the following formula:

Change in absorbance ($\Delta A_{Analysis}$) = $A_{Analysis}$ -$A_{Analysis; Blank}$

2. The residual absorbance ($\Delta A_{CLT; Blank}$) of the plant extract/drug independent from the inhibition of the β-hematin complex was calculated using the following formula:

$\Delta_{ACLT; Blank} = A_{CLTBlank} - A_{CLTBlank; Blank}$

3. The resulting inhibition of the β-hematin synthesis induced by the plant extract/drug was calculated using the following formula:

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

A positive $I_{Analysis}$ is considered as positive (active sample) whereas a negative value indicated a negative result[47].The IC_{50} values were means ± standard deviation of three independent experiments. Mann-Whitney U test was used to compare the mean IC50 of the plant extracts with that of chloroquine. P-value < 0.05 was considered significant.

RESULTS

The $I_{Analysis}$ values of methanol extracts of the 20 medicinal plants at a fixed concentration of 0.69 mg/mL varied and ranged from -0.002 to 0.39 (Table 1). At this fixed concentration, nineteen (19) out of the 20 methanol extracts showed positive $I_{Analysis}$ values. Only methanol extract of leaf of *Ocimum gratissimum* showed negative $I_{Analysis}$ value (-0.002) signifying inability to inhibit the formation of β-hematin). Methanol extract of *Alstonia boonei* stem bark displayed the highest $I_{Analysis}$ value of 0.39 while methanol extract of Sida acuta displayed the lowest $_{IAnalysis}$ value of 0.06 at a fixed concentration of 0.69 mg/mL. In addition, the $I_{Analysis}$ value of the standard drug chloroquine was 0.17 (Table 1).

Table 1. Percentage Yield and Beta-Hematin Inhibition of Twenty Selected Medicinal Plant Extracts

S/N	Plants/Drug	Family	Percentage Yield (%)	$I_{analysis}$ (0.69 mg/mL)
1.	*Morinda lucida*	Rubiaceae	23.0	0.11
2.	*Terminalia catappa*	Combretaceae	9.2	0.18
3.	*Ocimum gratissimum*	Lamiaceae	7.0	-0.002
4.	*Cajanus cajan*	Fabaceae	9.6	0.15
5.	*Vitex doniana*	Lamiaceae	6.4	0.13
6.	*Gossypium barbandense*	Malvaceae	7.3	0.17
7.	*Azadirachta indica*	Meliaceae	12.2	0.12
8.	*Mangifera indica*	Anacardiaceae	13.1	0.21
9.	*Phyllantus amarus*	Phyllanthaceae	6.3	0.24
10.	*Senna siamea*	Fabaceae	10.1	0.09
11.	*Lawsonia inermis*	Lythraceae	22.2	0.26
12.	*Nicotiana tabacum*	Solanaceae	18.6	0.21
13.	*Xylopia aethiopica*	Annonaceae	28.7	0.10
14.	*Allium sativum*	Amaryllidaceae	3.0	0.10
15.	*Euphorbia hirta*	Euphorbiaceae	11.7	0.07
16.	*Vernonia amygdalina*	Asteraceae	15.9	0.12
17.	*Momordica charantia*	Cucurbitaceae	9.2	0.26
18.	*Cymbopogon citratus*	Poaceae	3.2	0.19
19.	*Sida acuta*	Malvaceae	9.8	0.06
20.	*Alstonia boonei* *	Apocynaceae	5.2	0.39
21.	Chloroquine+	NA	NA	0.17

* With the exception of *Alstonia boonei* where the stem bark extract was tested, the methanol extract of leaves of the other medicinal plants were evaluated, +Standard antimalarial drug, NA – not applicable.

In addition, the 50% inhibitory concentration (IC_{50}) of the 6 most active medicinal plants from the initial screening was ($I_{Analysis} \geq 0.21$) quantitatively tested on the β-hematin assay at concentrations ranging from 0.04 to 1.38 mg/mL. Of the six methanol extracts tested, five possessed higher inhibitory activity greater than the standard drug chloroquine. Methanol extract of *Alstonia boonei* was the most ac-

tive with IC_{50} value of 0.09 ± 0.03 mg/mL while the extract of *Nicotiana tabacum* was the least active with IC_{50} value of 0.49 ± 0.05 mg/mL (Table 2). The mean IC_{50} value of chloroquine a potent β-hematin inhibitor was 0.36 ± 0.16 mg/mL (Table 2).

Table 2. Fifty Percent Inhibitory Concentration (IC_{50}) of Ten Medicinal Plants in a Beta-Haematin Inhibition Assay

S/N	Plant Extracts/Drug	IC_{50} (Mean ± S.D) (mg/mL)
1	*Alstonia boonei*	0.09 ± 0.03*
2	*Momordica charantia*	0.11 ± 0.02*
3	*Phyllantus amarus*	0.19 ± 0.03
4	*Lawsonia inermis*	0.24 ± 0.05
5	*Mangifera indica*	0.25 ± 0.07
6	*Nicotiana tabacum*	0.49 ± 0.05
	Chloroquine	0.36 ± 0.16

N = 3, *Chloroquine vs *Alstonia boonei* and *Momordica charantia* P<0.05

DISCUSSION

Host haemoglobin is an essential source of amino acids for parasite growth[20,21] for intraerthrocytic stages of malaria parasite i.e. rings, trophozoites and schizonts[20-22]. The food vacuole of the parasites contains specialised aspartic and cysteine proteases. These enzymes degrade the protein component of haemoglobin to amino acids, which are utilised by the parasite for protein synthesis[23-24]. The remaining part of the degraded haemoglobin is the haem (ferriprotoporphyrin IX), which is released intact. The haem produced along with amino acids after degradation of host haemoglobin is toxic to the parasites. Haem is membrane toxic, capable of lysing both the parasites and the red blood cells[25]. The parasites detoxified haem by converting it to an insoluble, unreactive crystalline material called haemozoin (malaria pigment) or β-haematin[26].Inhibition of conversion of toxic haem to β-haematin by 4–aminoquinolines or the complex formed by toxic haem and some antimalarial drugs would effectively allow the build-up of haem to a level that become irreversibly toxic to the parasite. Thus, the haemozoin formation pathway has been widely studied and explored as the mechanistic pathway for the 4-aminoquinolines antimalarial drugs such as chloroquine, mefloquine and amodiaquine13-15. This pathway is also considered a suitable target for antimalarial drug discovery[27-29].

The β-haematin formation pathway was explored in this study to evaluate the mechanism of antimalarial activity of twenty medicinal plant species from sixteen different families reputed to be of importance in ethnomedicine for the treatment of malaria. Nineteen out of the twenty plant extracts showed positive $I_{Analysis}$ values which signified activity against β-hematin synthesis. Two of the plant extracts *(A. boonei and M. charantia)*, showed significant activity than chloroquine (P<0.05). Others active plant extracts were *P. amarus, L. inermis, M. indica* and *N. tabacum*. These plants showed higher inhibition of β-hematin synthesis than chloroquine a known β-hematin synthesis inhibitor. The previously reported antimalarial activity of *A. boonei*[30], *M. charantia*[31-33] *P. amarus*[34], *L. inermis*[30], and *M. indica*[35] in a parasite based assay using *in vitro P. falciparum* might due to inhibition of β-hematin synthesis in the parasites. It appears there is no information on antimalarial activity of *N. tabacum*. However, larvicidal, mosquitocidal and antioxidant activities of *N. tabacom* were previously reported [36-37] The mechanism of antimalarial activity of these plant species from sixteen different families vis-a-vis inhibition of β-hematin synthesis is being reported for the first time.

CONCLUSION

The β-hematin colorimetric assay is a reliable assay for determining the mechanism of action of antimalarial agents that utilize the pathway.

Both authors reviewed and approved of the final manuscript.

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CONFLICT OF INTEREST

We declare no conflict of interest.

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