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Effect of *Senna occidentalis* (Fabaceae) leaves extract on the formation of β-hematin and evaluation of *in vitro* antimalarial activity

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Abstract
The search for antimalarials from plant sources has yield significant success in drug discovery approaches. The heme polymerization inhibitory activity as well as the antimalarial activity of *Senna occidentalis*, a local medicinal plant used for malaria therapy in Hausa folk medicine in Northern Nigeria was evaluated in *in vitro* assays. Results obtained revealed a good inhibition of *β*-hematin formation (83.08% and 83.97%) by the methanolic and aqueous leaves extracts of *S. occidentalis* at 500µg/mL as against the 54.92% inhibition exhibited by hexane extract at the same concentration. Findings of the *in vitro* antimalarial studies revealed a dose dependent suppression of plasmodium growth. At a concentration of 6.25 µg/ml, 73% suppression of parasite growth was observed for the hexane extract. This suppression of plasmodial growth attains 84.43% at 50 µg/ml with an IC_{50} of 3.47 µg/ml. Secondary metabolites such as anthraquinones, phenols, tannins, alkaloids and flavonoids were detected in *S. occidentalis.* This could be a responsible for the antimalarial activities observed.

Keywords: Heme, β-hematin, antimalarial, *Senna occidentalis*, medicinal plants

Introduction
The use of natural products, in particular, medicinal plants for the treatment of a number of disease conditions is as old as mankind. *Senna occidentalis*(Fabaceae) commonly called coffee senna (English), Majamfari/Bazamfari/Tafasar Masar (Hausa), is an annual shrub with characteristic yellow flower and dehiscent woody pod seeds found in water canals, open dumping sites and by roadsides [1]. In Hausa folk medicine, *S. occidentalis* is used for the treatment of various ailments including but not limited to; Typhoid fever, malaria and hepatitis in Northern Nigeria [1, 2]. Oral intake of a decoction of fresh leaves of *S. occidentalis* or steam bath may be used to cure fever [2]. Similarly, the plant was reported to possess larvicidal and mosquitocidal activities [3], antioxidant and antimicrobial [4], anti-inflammatory, immunosupressive, antianxiety, antidepressant, analgesic, antidiabetic and antipyretic activities [5].

For malaria chemotherapy to be precise, the feats recorded from plant products (Cinchona and *Artemisia annua*) is worth mentioning [6]. Most antimalarial drugs in clinical use target the blood schizonticidal stage of malaria parasite. Schizonticidal antimalarials either prevent the formation of haemozoin (malaria pigment) [7], a pathway that leads to unwarranted increase and accumulation of free hemein the parasites digestive vacuole, thus, generating reactive oxygen species, hinder the activities of important enzymes, lyse vacuolar membrane and eventually cause parasite death [8], or interfere with folate biosynthesis [9] a principal player in parasite metabolism. Some schizonticidal antimalarials are known to impede gametogenesis [10] resulting in blockage of further transmission. On the other hand, the 8-aminoquinolines (primaquine) target the liver (hepatic schizont) stage, especially in relapse forming parasites and early developmental stages [11].

It is worthy of note however, that the potency of many antimalarials is essentially related to their abilities to inhibit hemozoin (β-hematin) formation [12]. Despite the availability of synthetic drugs for malaria in clinical use, inhabitants of rural settings, the less previledged and the poor rely mostly on herbs and other forms of traditional medicines to treat the disease [13, 14]. This may not be unconnected to their lack of access to health facilities coupled with financial constraints [14]. To achieve total elimination of the deadly parasite that has claimed millions of lives, the search for new antimalarials with broad specificity and novel mode of action from plant sources is imperative. In this study, we evaluate the abilities of hexane, methanol and aqueous leaves extracts of *S. occidentalis* to prevent β-hematin formation *in vitro*.

The antimalarial efficacies of these extract on the chloroquine sensitive (CQS) strain (3D7) of *P. falciparum* was also ascertained *in vitro.*

Material and Methods

Chemicals and reagents

Bovine haemin and Sodium acetate were purchased from Molekula, UK, chloroquine diphosphate ($CQdiPO_4$) was from Sigma Aldrich, UK, while acetic acid (glacial) (J.T. Baker) and dimethylsulfoxide (DMSO) (Sigma Aldrich).

Plant identification

Senna occidentalis (whole plant) was identified by a plant Taxonomist in the Department of Biological sciences, Yobe State University, Damaturu. Voucher specimen was deposited in the herbarium of the same department for reference purposes.

Phytochemical extraction

Fresh leaves of *S. occidentalis* was hand-picked, washed in tap water and shade dried at ambient temperature [15] to avoid loss of active metabolites. Dried leaves were grinded by milling machine (IKA WERKE, M20) to form a coarse powder. Successive extraction of bioactive compounds was performed using n-hexane, methanol and distilled water respectively. One hundred grams (100g) of the powdered plant material was weighed and macerated in 500ml (1:5 w/v) n-Hexane (JHD, Guangzhou, China) and maintained by constant shaking for 24 hours in a shaker (IKA WERKE, HS501). This was then filtered using muslin cloth and later with Whatmanno.1 filter paper. The filtrate was concentrated using rotary evaporator at 40 ºC (Stuart, RE 300 DB), while the residues were air dried and used for further extraction with methanol and distilled water respectively as described. Aqueos extracts were free zed at -20 ºC and lyophilized in a freeze drier (FDL-10N-50-TD-MM). Subsequent to extraction of active metabolites, each extract was weighed and the percentage yield was calculated using the following formula

$$\text{Percent (\%) yield} = \frac{\text{Weight of extract (g)}}{\text{Weight of Powdered Material (g)}} \text{ X } 100$$

Phytochemical screening

Test for Phenols: To 1 ml extract, 2 ml distilled water was add followed by few drops of 10% aqueous ferric chloride. Blue or green colouration indicates the presence of phenols

Test for Tannins: plant extract (0.25g) was weighed and dissolved in 10 ml distilled water, then filtered. To the filtrate, 1% aqueous Iron chloride (FeCl3) solution was added. Intense green, purple, blue or black colouration indicates the presence of tannins.

Test for Anthraquinones (Borntrager's Test): plant extract, (2g) was weighed and dissolved in petroleum ether and filtered. To the filtrate, aqueous ammonia was then added. Pink colouration indicates the presence of anthraquinones.

Test for Alkaloids: To 0.5g of extract, 8 ml of 1% HCl was added, warmed and filtered. The filtrate was treated separately with 2 ml of Mayer's and Dragendorff's reagents. Formation of turbidity or precipitate indicates the presence of alkaloids.

Test for Flavonoids: To 0.5 g of extract, few drops of petroleum ether was added and shake to remove the fatty materials (lipid layer). The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. To 3 ml of the filtrate, 4 ml of 1% potassium hydroxide was added in a test tube. A dark yellow colouration confirms the presence of flavonoids.

β-hematin assay

Powdered plant extracts were weighed and dissolved in dimethylsulfoxide (DMSO) to ensure complete dissolution and reconstituted in distilled water to obtain different concentrations [4]. A stock; hemin chloride (6.5 mM), sodium acetate (3M), Chloroquine diphosphate (0.8mM), and different concentrations of crude plant extracts were prepared. In this assay, 50µl of freshly prepared hemin chloride (6.5 mM in 40% DMSO), 100 µl of sodium acetate (3M), 25 µl of glacial acetic acid (17.4M) (pH 3.8-5.2) and 25 µl of test compound (final concentration in respective wells 500, 250, 125 and 62.5 µg/mL)or chloroquine diphosphate(final concentration in respective wells 31.25, 15.625, 7.8125 and 3.90625nM/mL) to serve as positive control) were added into duplicate wells of a sterile 96 wells flat bottom plate and incubated for 24 hours at 37 ºC. It is important that the mixture is added in the order stated [16, 24, 25].Following incubation, the plate was centrifuge at 13000 rpm for 15 min, the supernatant discarded and the pellet resuspended in 200 µl (DMSO) to remove free hemin chloride [16]. Washing was repeated 3-5 times until a clear supernatant is obtained. Consequently, formed pellets were dissolved in NaOH (0.1M) the only compound reported to break the iron-carboxylate bond and convert β-hematin to heme [17], resuspended (1 in 10) in NaOH(0.1M) and absorbance obtained at 405nM in a microplate reader.

Percentage inhibition of hemin polymerization by test compounds was calculated using the following formula; [24].

$$\text{Percentage inhibition (\%I)} = \frac{\text{M. Abs (placebo control)} - \text{M. Abs (test compound)}}{\text{M. Abs (placebo control)}} \text{ x } 100$$

Where M. Abs. = Mean Absorbance.

Parasite culture

Parasites were maintained in RPMI medium 1640 (gibco) supplemented with sodium bicarbonate, glucose, Hypoxanthine, gentamicin, L-glutamine and Albumax. Cryopreserved chloroquine sensitive (CQS) *P. falciparum* (3D7) was maintained in continuous culture according to the methods ofMoon *et al.* [18] with little modifications. Briefly, cryovials containing parasitized erythrocytes (iRBCs) were thawed for 5 minutes in a water bath set at 37 ºC. Volume of iRBCs was measured, transferred to 15ml falcon tube and equal volume (1:1 v/v) of thawing solution (3.5% NaCl) was added slowly, dropwise, while shaking gently [19]. The mixture was repeatedly washed 3 times with equal volume of culture medium until a clear supernatant is obtained [19]. The supernatant was discarded and the final volume of iRBCs was measured and transferred to a sterile culture flask. Equal volume of washed RBCs (group O+) and a required volume complete culture medium (CM) was added, gassed (88% Nitrogen, 7% Carbon dioxide and 5% Oxygen) and incubated at 37 ºC. Parasitaemia was monitored and medium changed daily [20].

In vitro antimalarial sensitivity assay

Histodenz synchronised (rings and trophozoites) *P. falciparum* (3D7) cultures was used. Briefly, a sterile 96 wells flat bottom plate was used for this assay, with respective wells

receiving 180 µL of CQS culture (1% parasitaemia, 2% haematocrit) and 20 µL of test compound (final concentration in respective wells; 50, 25, 12.5 and 6.25 µg/mL), chloroquine (final concentration 1000, 500, 250, 125, 62.5, 31.25 and 15.625 nM) or dH_2O. Plates were placed gas chamber, gassed (88% Nitrogen, 7% Carbon dioxide and 5% Oxygen) and incubated at 37 ^oC for 48 hours. Gassing was repeated after the first 24 hours and gas chamber placed back in the incubator for another 24 hours.

Subsequently, parasites were harvested as previously described [21]. Thin smears were prepared in grease free glass slide, fixed with methanol, allowed to air dry and stained with 10% giemsa in PBS for 20 minutes. Later, the slides were gently rinsed in running tap water and dried with a low-power hair dryer, and read at 100x magnification under oil immersion in a microscope (OlympusCX22LED) [22].

Percentage parasitaemia and growth suppression was calculated using the formulae:

$$\text{Percentage parasitaemia} = \frac{\text{Number of infected Red Blood Cells (iRBCs)}}{\text{Total number of Red Blood Cells (RBCs)}} \text{ X } 100$$

$$\text{Growth suppression (\%)} = \frac{\text{Mean parasitaemia (Negative control) - Mean parasitaemia (Treated group)}}{\text{Mean parasitaemia (Negative control)}} \text{ x } 100$$

Statistical Analysis

Data obtained was analysed using Microsoft excel 2010 and then Graphpad prism 5.0. For IC_{50} calculations, data was normalised so that the largest value in the data set corresponded to 100% and the smallest value corresponded 0%. Log-transformed drug/crude extract concentrations were then plotted against the dose response and the IC_{50} values were determined using nonlinear regression. The log (inhibitor) vs. Normalised response- Variable slope option was selected for this analysis.

Results and Discussion

The yield (%) obtained for the three solvent extracts of *S. occidentalis* was in the range of 31.63–8.95, with the highest percentage yield observed in n-hexane extract (31.63%) and the lowest from aqueous extract (8.95%) (Table 1).

Table 1: Yield of Hexane, Methanol and Aqueous crude leaves extracts of *S. occidentalis*.

Plant	Part used	Extract	Weight of powder (g)	Yield (g)	Yield (%)
S. occidentalis	Leaves	Hexane	500	158.14	31.63
		Methanol	500	61.48	12.29
		Aqueous	500	44.73	8.95

Results of phytochemical analysis of three solvent extracts of *S. occidentalis* are presented in table 2 below. Findings revealed the presence of phenols, tannins, anthraquinones and flavonoids in all solvent extracts with the exception of lyphilized aqueous extract in which alkaloid was not identified. This finding corroborate with the result of Musa *et al.* [23].

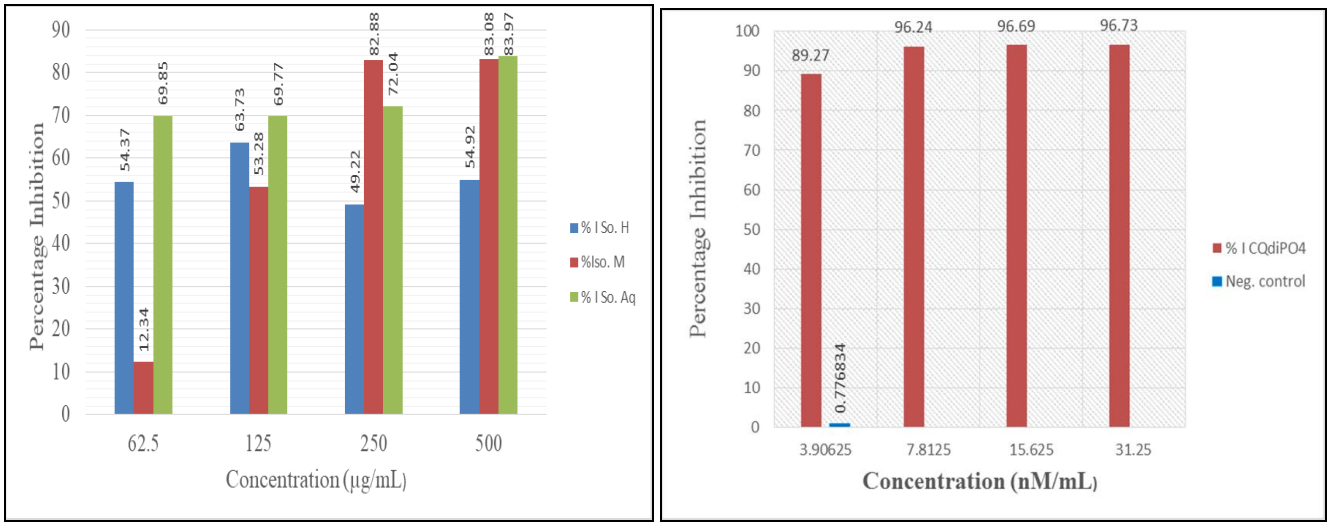
Table 2: Phytochemicals identified in Hexane, Methanol and Aqueous crude leaves extracts of *S. occidentalis*.

Plant	Phytochemicals	Type of Extract		
		Hexane	Methanol	Aqueous
S. occidentalis	Phenols	+	+	+
	Tannins	+	+	+
	Anthraquinones	+	+	+
	Alkaloids	+	+	-
	Flavonoids	+	+	+

Key: + = present; - = absent

The inhibition of haemozoin formation is an attractive target for development of several antimalarial drugs and is considered a suitable target for drug screening programs. Results presented (Figure 1), are percentage inhibition of heme polymerization calculated from the absorbance readings obtained. Absorbance values had been previously reported to be inversely proportional to efficacy of test compounds or drugs, inferring higher efficacy, when the absorbance are low and vice versa [8, 16, 24-25]. In this study, a relatively good inhibition of β-hematin formation (83.08% and 83.97%) was revealed by Methanolic and aqueous leaves extracts of *S. occidentalis* at 500µg/mL as against the 54.92% inhibition exhibited by hexane extract at the same concentration(Figure 1a). Although a good activity is expressed by Methanolic and aqueous leaves extracts, this may not be relied upon as baseline that defines the exact pathway targeted by this plant in the malaria parasite, if at all, it has potent antimalarial properties. This assertion corroborate with a recent finding insinuating that false negative compounds may show their anti-plasmodial potencies via other modes of action [26]. Notwithstanding, this method is however, a good predictor for screening antimalarial activity in compounds capable of interacting with heme. At the lowest concentration (62.5 µg/mL), a very poor (12.34%) inhibition was demonstrated for the crude methanolic extracts of *S. occidentalis.* This activity increases (53.28%, 82.88% and 83.08% respectively) as the concentration increases. This finding may not be unconnected with the presence of anthraquinones in the methanolic extracts of *S. occidentalis* as reported in this and other study [4]. The ability of quinolines to form complex with heme in an acidic environment symbolizes its effect in parasite death. Similarly, the presence of squalene, a nutral antioxidant in the hexane and methanol extract of *S. occidentalis* may be responsible for the efficay observed [27, 28] as compounds with antioxidant properties show potent antimalarial properties [29]. At nano molar (nM) concentrations, it is not surprising that the reference antimalarial tested as positive control inhibited heme

detoxification by approximately 90% (Figure 1b) at the lowest concentration (3.90625nM/mL) and ≥97% at the highest concentration (31.25 nM/mL). This activity may be attributed to the complex formed when free heme binds to chloroquine in the acidic food vacuole, thereby interfering with heme detoxification [17, 33-34].



(A) (B)

Fig 1: Effect of Hexane, Methanolic and Aqueous leaves extracts of *Senna occidentalis*, Chloroquine diphosphate and distilled water on the formation of β-hematin

Sensitivity tests were carried out to establish the effect of crude *S. occidentalis* (Leaves) extracts against chloroquine sensitive *P. falciparum* (3D7) culture *in vitro*. Table 3 below summarizes the percentage parasitaemia, percentage suppression of parasite growth and the IC_{50} of extracts investigated. Results are presented as Mean ± Standard error of mean (M±SEM) except for the IC_{50}.

Table 3: *In vitro* antimalarial effect of Hexane, Methanol and Aqueous leaves extracts of *S. occidentalis* and the reference antimalarial against *P. falciparum* (3D7)

Type of extract	Dose (µg/mL)	Parasitaemia (%) Mean ± SEM	Growth suppression (%)	IC_{50} (µg/mL)
Hexane	6.25	1.95 ± 0.12	72.52	3.47
	12.5	1.72 ± 0.04	75.72	
	25	1.44 ± 0.06	79.61	
	50	1.10 ± 0.03	84.43	
Methanol	6.25	2.01 ± 0.13	71.60	3.79
	12.5	1.39 ± 0.10	80.44	
	25	1.09 ± 0.20	84.65	
	50	0.72 ± 0.16	89.81	
Aqueous	6.25	3.05 ± 0.16	56.94	4.03
	12.5	2.32 ± 0.10	67.30	
	25	2.05 ± 0.08	71.07	
	50	1.76 ± 0.27	75.13	
CQ (nM/mL)	15.625	2.85 ± 0.59	59.84	11.79
	31.25	1.55 ± 0.03	78.12	
	62.50	0.74 ± 0.06	89.63	
	125	0.68 ± 0.1	90.40	
	250	0.41 ± 0.09	94.28	
	500	0.21 ± 0.05	97.03	
	1000	0 ± 0	100	
CM only	20 µL	7.08±1.39	-	-

Key: CQ = Chloroquine (Positive control); CM only = Culture Medium only (Negative Control)

Findings of the *in vitro* studies of crude hexane, methanol and aqueous leaves extracts (Table3) show that; for a concentration of 6.25 µg/ml, the hexane extract inhibited parasite growth by 73%. This suppression of plasmodium growth is dose dependent and attains 84.43% at 50 µg/ml with an IC_{50} of 3.47 µg/ml. When tested against 3D7 strain of *P. falciparum*, Murugan *et al.* [3] reported a somewhat higher IC_{50} (48.80 µg/mL) for ethanolic extracts of *S. occidentalis* and 54.28 µg/ml on chloroquine resistant INDO strain. Studiesof the methanol extracts in this research revealed about 90% reduction in parasite growth at the highest dose level (50 µg/mL), and an IC_{50} of 3.79 µg/ml. In Democratic Republic of Congo (Congo D.R.), Kayembe *et al.* [30] isolated six quinones from crude ethanol extract of *S. occidentalis* leaves. The crude ethanol extract showed potent antimalarial activity against *P. falciparum in vitro*, presenting 93% suppression of parasite growth at 25 µg/mL concentration. As for the lyophilised aqueous leaves extract of *S. occidentalis*, 57% growth suppression was observed for the lowest concentration extract, while 75% inhibition was obtained at the highest dose

(IC_{50} 4.03 µg/ml). These findings revealed that; the lyophilised aqueous leaves extract is less active when compared with the methanol and hexane extract. This conforms to the result previously reported [31]. It is not surprising that different IC_{50} values were obtained for same plants. This disparity may perhaps be associated to geographical locations, growing conditions of the plants as well as variation in extraction procedures. This is specifically true for *S. occidentalis*. For example, in Congo D.R., Tona*et al*.[32]re-counted potent antimalarial activity of the ethanol leaf extract of *S. occidentalis* against 3D7 strain of *P. falciparum, in vitro* with an IC_{50}<3 µg/mL.

When assayed at nM concentrations, the reference antimalarial showed ≥60% suppression of plasmodial growth at the lowest concentration (15.63 nM/mL). The suppression of plasmodial growth was observed to be directly proportional to drug concentration. As the concentration increases, so does the inhibition of parasite growth. At 125 nM/mL ~90% growth suppression was noticed while total clearance was observed at the highest dose 1000nM/mL. This finding further suggest that a much lower concentration comparable to the lowest concentration is required to prevent the growth of parasites by 50% (IC_{50} = 11.79nM/mL).

Conclusion

Lack of malaria vaccine in clinical use, resistance to frontline antimalarials and reliance of the less privileged on herbal remedies to treat the disease made it necessary to search for new antimalarials with novel modes of action. Here we determine the therapeutic potentials of a commonly used medicinal herb for malaria in Northern Nigeria. We recommend further studies to determine the antimalarial efficacies of this herb *in vivo*.

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Conflict of Interest: The authors declare that we do not have conflict of interest regarding this manuscript.

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