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HEMIN POLYMERIZATION INHIBITORY ACTIVITIES (HPIA) AND *in vitro* ANTIMALARIAL PROPERTIES OF CRUDE *Senna siamea* LEAVES EXTRACT ON *P. falciparum*, 3D7 STRAIN

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ABSTRACT

Over the last two decades, malaria parasites have been reported to develop clinical resistance to frontline antimalarials recommended for use in malaria endemic parts of the world. This resistance to antimalarial drugs has led to the death of many promising infants, expecting mothers and immunocompromised individuals in tropical Africa. This research assesses the ability of crude hexane, methanolic and aqueous *Senna siamea* leaves extract to inhibit haem polymerization in an *in vitro* assay. Similarly the antimalarial potential of the plant was determined in *P. falciparum* (3D7) *in vitro.* Results obtained revealed that the methanolic leaves extract inhibits heam detoxification by 90% at 500 µg/ml concentration and by 56% at 62.5 µg/ml. Similarly, the aqueous extract suppresses parasite growth by 81% at 50 µg/mL with an IC_{50} value of 5.10 µg/mL. These good antimalarial activities exerted by crude *Senna siamea* extracts may be as a result of the presence of essential phytochemicals; Anthraquinones, Alkaloids, flavonoids, tannins observed in both solvent extracts. Based on the findings of this study, it could be concluded that both extracts possess good antimalarial potentials.

Keywords: Malaria, Hemin, Antimalarial, *Senna siamea*

INTRODUCTION

In sub-Saharan Africa, Malaria accounts for 241 million cases with 80% mortality occurring in infants <5 years of age (WHO, 2021). This is almost the leading cause of death in immunocompromised individuals. The disease is caused by parasites of the genus Plasmodium. Five species; *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* were reported to infect human in tropical and subtropical regions of the world. Of these, *P. falciparum* is the most virulent while *P. vivax* is the most widespread (Larson, 2019). The parasites are transmitted to human by the bite of an infected female *Anopheles* mosquito. In rare occasions, *Culex* mosquitoes also transmit the disease. As part of conditions to ensure continuity of species, the newly emerged female mosquito had to select the fittest male in swamp for mating, following which no mating occurs as the uterine opening is sealed by mating plugs (Diabate and Tripet, 2015; Daskum *et al.,* 2019). However, these mosquito lays several batches of eggs during their life cycle. For egg development, the female mosquito takes a blood meal from human or other species-specific vertebrate host (Marques*et al.,* 2018). During blood meal, the naïve mosquito acquires the infective stage (gametocytes) of the parasite from infected individual (Service, 2008). These further underwent sexual development that result in the production of sporozoites (developmental stages that infect human) which coincides with the maturity of eggs ready for laying (Gunn and Pitt, 2012). Following oviposition, the female mosquito requires another blood meal to develop the second batch of her eggs, and so on. During blood feeding, the infective stages (sporozoites) are injected into the blood stream, which marks the beginning of another development (asexual) of these parasites in the human. It imperative to note that the development cycles which alternates between human and mosquito involves feeding and non-feeding stages. In the mosquito, the parasite is non-feeding but feeds on blood while in the human host.

In the vertebrate/human host, the parasite undergoes two developmental cycles (in the liver cells and the red blood cells), both producing merozoites, infective on healthy erythrocyte or transforming to gametocytes (Gunn and Pitt, 2012). While in the erythrocytes, the parasite feeds on the oxygen-carrying pigment (haemoglobin), degrades it incompletely by breaking it down into two (haem and globin) component in the parasites' food vacuole, Figure 1 (Goldberg and Zimmerberg, 2020) due to its lack of the enzyme; haem oxygenase (Moore *et al.,* 2006).

While the globin component is essential for parasite growth and development, the haem component is toxic and affects the parasites by lysing the cell membrane, releasing the cytosol component hence resulting in parasite death (de Villiers and Egan, 2021). Continuous degradation of haemoglobin leads to excessive accumulation of lethal haem in the parasites' food vacuole. To overcome the toxicity of free haem, the parasite converts them into non-toxic haemozoin, otherwise termed malaria pigment (Ramya *et al.,* 2002).

Most antimalarial drugs, for example the quinolines and artemisinins exert their effects by preventing the conversion of free haem to haemozoin and hence allows for excessive accumulation of free heam that kills the parasite (Herraiz *et al.,* 2019). While the malarial parasites have developed clinically ample resistance to most of the antimalarial drugs targeting the haem pathway, the search for antimalarial drugs from plant sources that might inhibit conversion of haem to haemozoin (haem/haemin pathway) is essential.

The antimalarial potential of *Senna siamea* (leaves, roots and stem bark) have been well documented in the literature(Ekasari *et al.,* 2021; Adukpo *et al.,* 2020; Tasiam *et al.,* 2020; Javeres *et al.,* 2019). Despite this, only few research considers target specific approach to ascertain the potentials of medicinal plants. However, little is known about the target pathway of this plant in the malarial parasite. This research assesses the ability of the crude hexane, methanolic and

aqueous *Senna siamea* leaves extract to inhibit haem polymerization in an *in vitro* assay.

MATERIAL AND METHODS

Plant collection and identification

The plant was identified by its local name. Leaves, fruits and flowers were collected and further identified and classified according to Taxonomic keys by a plant taxonomist in the herbarium, Department of Plant Science, Modibbo Adama University, Yola, Nigeria. Accession number (SA 044) was assigned, and voucher specimen (015) was deposited in the herbarium for reference.

Phytochemical Extraction

Prior to phytochemical extraction, fresh leaves were collected and transported to the Zoology research laboratory, Department of Zoology, Modibbo Adama University, Yola, Nigeria. Samples were washed in running tap water and dried under shade. Dried leaves were further ground to powder in a wooden pestle and mortar and sieved to obtain fine powder. Powdered specimen was then transferred into Bama bottle, clearly labelled, capped and stored until use.

Successive extraction with solvents (n-hexane, Methanol, and distilled water) in order of increasing polarity was perfromed as per Daskum *et al.* (2020) to extract all active ingredients based on solvent polarity. Briefly, samples were weighed such that100 g powdered plant material was transferred into screw cap Bama bottles and macerated in 500 ml each of organic solvent (i.e. 1:5 w/v). The suspension was thoroughly stirred with a glass rod, capped and shaken for 24 hours in a shaker (IKA WERKE, HS 501). This was later filtered, residues air dried and used for further extraction with a more polar solvent. Filtrates of n-hexane and methanolic extractions were concentrated at 40°C using a rotary evaporator (Stuart, RE300DB) and solvents recovered, while those of aqueos extracts were freezed to ice and later freeze dried in a lyophilizer (FDL-10N-50-TD-MM).The weight of individual extract was determined and the percentage yield calculated

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

Phytochemical Screening

Following phytochemical extraction, crude extracts were subjected to phytochemical screening, with a view to determining the presence or otherwise of some active metabolites. For the analysis of phytochemicals, qualitative test was performed in accordance with the protocol described by Kumar *et al.* (2013).

Haem Polymerization Assay

To ascertain the effect of crude *Senna siamea* leaves extract on haem polymerization, we mimicked the parasite food vacuole in an*in vitro*assay. However, parasite lysate was not used throughout the assay. The assay condition was such that an acidic environment was formed. As described (Daskum et al., 2019),6.5 mM bovine hemin solution, 3 M sodium acetate, 0.8 mM chloroquine phosphate solution and 0.1 M NaOH solution was freshly prepared as the stock solution. These was used to make the reaction mixture, thus; to 1ml eppendorf tubes, 100 µL of freshly prepared bovine hemin, 50 µL of the test compound (crude *Senna siamea* leaf extracts) and 50 µl of 17.4 M glacial acetic acid (pH 3.8) was aliquoted incubated at 37°C for 24 hours. This was later washed by continuous centrifugation at 3500 g for 15 minutes, supernatant discarded and pellets resuspended in 200 µl DMSO. This was repeated until a clear supernatant is obtained. In accordance with Tekwani and Walker, (2005) pellets formed after the final wash suspected to be β-haematin/malaria pigment could be converted to hemin by dissolving in 0.1M NaOH for spectrophotometric quantitation of β-haematin as haem equivalents. Before taking absorbance reading for spectrophotometric quantification of β-haematin solution, 1ml of 0.1M NaOH was aliquoted onto a plastic cuvette and a blank measurement was obtained in a UV-Visible spectrophotometer. Subsequent to this, absorbance reading set at 405nM was obtained for all assay groups.

Percentage inhibition of hemin polymerization by test compounds was calculated using the following formula; (Mojarrab *et al.,* 2014).

$$\text{Percentage Inhibition (\%I)} = \frac{\text{(Normal activity – Inhibited activity)}}{\text{Normal activity}} \times 100$$

$$\text{\% I} = \frac{\text{(Mean Absorbance of placebo control-Mean Absorbance of test compound)}}{\text{Mean Absorbance of placebo control}} \times 100$$

Parasite Culture

Parasite culture media was prepared as per Moon *et al.* (2013). Isolates were maintained in blood (O+) in the tissue and malaria culture laboratory, Biology Research laboratory, Department of Biological Sciences, Yobe State University, Damaturu. Prior to parasite culture, blood obtained from voluntary donors was washed by centrifugation and resuspended in culture media (50% haematocrit) right after the last wash before storage at 4°C until use.

Cryopreserved parasites isolates were thawed on the bench and later transferred into the biosafety cabinet. Volume of isolate was measured, aliquoted and 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 (Amir, 2016). This was centrifuged at 1000 rpm for 5 minutes and supernatant discarded. This process was repeated until a clear supernatant is obtained and the final volume of iRBCs was measured and transferred to a culture flask. Cultures were maintained and synchronized in accordance with Daskum *et al.* (2019).

Antimalarial Sensitivity Assay

A stock (100mg/ml) of crude hexane, methanolic and aqueous *Senna siamea* leaves extract was freshly prepared according to the methods of Donkor *et al.* (2015). An aliquot (10 µL) of the stock was reconstituted in 990 µL of culture medium to attain a concentration of 1000µg/ml, which was serially diluted to obtain various concentrations (500, 250, 125 and 62.5 µg/ml) respectively. Similarly, a1000nMchloroquine phosphate (1:1 v/v stock in 70% ethanol) stock was also prepared and serially diluted to obtain (125, 62.5, 31.25 and 15.625 nM) working solutions, respectively. To a 96 wells flat bottom plate, 25 µL of the chloroquine phosphate was transferred to duplicate wells columns (11 and 12) and allowed to stand overnight (air dry) in a biosafety cabinet. Following coating, 180 µL of synchronized parasite cultures (1% parasitaemia, 2% haematocrit) were seeded to duplicate wells (columns 1-10). Subsequent to seeding, 20 µL of crude plant extracts was transferred to duplicate wells(columns 1-8) to yield a final concentration (50, 25, 12.5 and 6.25 µg/ml) while 20 µL of the culture medium was transferred to columns

9 & 10 to serve as negative control. It should be noted that columns 3 and 6 were not treated with extracts or reference drugs.

To columns 11 and 12 earlier pre coated with chloroquine phosphate, 200 µL of synchronized parasite cultures was added to serve as positive control. Microplates were then sealed, gassed (in candle jar) (Trager and Jensen 1976) and incubated at 37°C for 48 hours. Gassing was repeated after the first 24 hours and gas chamber placed back in the incubator for another 24 hours.

Following incubation, treated parasite cultures were harvested, smeared, fixed with methanol, stained (10% giemsa in PBS for 20 minutes) and examined microscopically in accordance with Basco, (2007) for assessment of sensitivity of extracts.

Percentage parasitaemia and growth suppression was determined thus;

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

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

RESULTS

The yield of hexane, methanol and aqueous *S. siamea*leaves extract expressed as percentage yield (Table 1) was in the range of 10.49 -18.73%, withhexane extract having the highest (18.73%) percentage yield obtained.

Table 1: Yield of crude *S. siamea* leaves extracts

Plant	Local/Hausa Names (English)	Part used	Extract	Yield (g)	Yield (%)
S. siamea	Malga/marga (Kassod tree)	Leaves	Hexane	93.66	18.73
			Methanol	61.06	12.21
			Aqueous	52.49	10.49

Extracts subjected to phytochemical analysis reveals the presence of phenols, terpenoids, tanins, cardiac glycosides, Xanthoproteins, Alkaloids and flavonoids in extracts analysed. However, terpenoids and resins were not detected in the methanol and hexane extracts, respectively (Table 2).

Table 2: Phytochemical analysis of crude *S. siamea* leaves extracts

Type of extract Plant species	Phenols	Terpenoids	Resins	Tannins	Cardiac glycosides	Anthraquinones	Xanthoproteins	Saponins	Alkaloids	Flavonoids
n-Hexane										
S. siamea	+	+	-	+	+	+	+	+	+	+
Methanol										
S. siamea	+	-	+	+	+	+	+	+	+	+
Aqueous										
S. siamea	+	+	+	+	+	+	+	+	+	+

Bioactive metabolites screened, indicating; + for Present/identified and - for Absent/not identified

Results of Hemin Polymerization Inhibitory Activity (HPIA) performed in this study is presented as percent inhibition of heme polymerization exerted by crude plant extracts. Absorbance reading obtained was used to calculate percentage inhibition of hemin polymerization caused by crude plant extracts. The methanol extract of *S. siamea* inhibited hemin polymerization by 56.46% at the lowest dose and 89.62% at the highest concentration of crude extract while activity of the aqueous extract was in the range of 77.65% to 81.68% respectively, (Figure 2).

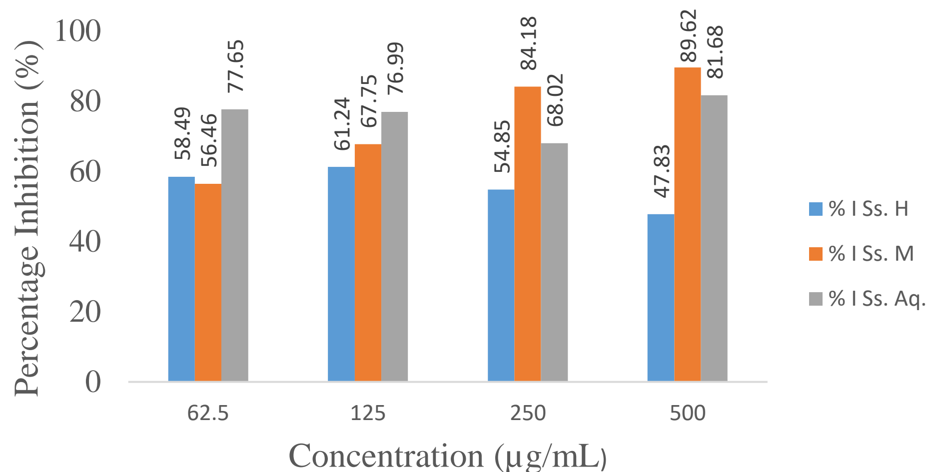

Figure 2: Percent inhibition of haem polymerization caused by crude *Senna siamea* leaves extract

To establish the effect of crude *S. siamea* (Leaves), antimalarial sensitivity was performed on chloroquine sensitive *P. falciparum* (3D7) culture *in vitro*. For extract and the reference antimalarial drug (Chloroquine), the percent suppression of parasite growth and IC_{50}were determined. Table 3 below summarizes the percentage parasitaemia, percentage suppression of parasite growth and the IC_{50} of extracts investigated. Results of parasitaemia are presented as Mean ± Standard error of mean (M±SEM) except for the IC_{50}, while percentage suppression of parasite growth was calculated as per the formula stated. A dose dependent suppression of parasite growth was observed for all extracts, with 81% suppression observed for the highest concentration (50 µg/mL) of aqueous extract and 64 and 53% suppressions of parasite growth observed for the methanolic and hexane extracts, respectively. Similarly, results showed that 4.35 µg/mL and 3.88 µg/mL of the methanolic and hexane extracts could be required to clear parasitaemia by 50%

Table 3: *In vitro* antimalarial activities of hexane, methanolic and aqueous *S. siamea* (leaves) extracts

Plant Name	Type of extract	Dose (µg/mL)	Parasitaemia (%)	Growth supression (%)	IC_{50} (µg/mL)
			Mean ± SEM		
S. siamea	Hexane	6.25	4.16 ± 0.04	41.32	3.88
		12.5	3.94 ±0.12	44.37	
		25	3.71 ±0.09	47.63	
		50	3.33 ± 0.28	52.97	
	Methanol	6.25	3.83 ± 0.25	46.00	4.35
		12.5	3.34 ± 0.26	52.83	
		25	2.90 ± 0.24	59.03	
		50	2.52 ± 0.03	64.43	
	Aqueous	6.25	3.44 ± 0.55	51.43	5.10
		12.5	2.69 ± 0.23	61.99	
		25	2.03 ± 0.07	71.29	
		50	1.36 ± 0.21	80.77	

SEM = Standard error of Mean; IC_{50} = 50% Inhibitory concentration µg/mL = Microgram per mills concentration

DISCUSSION

Important phytochemicals; Alkaloids, Flavonoids, Saponins, Steroids and Tannins were identified in *S. siamea* leaves extracts. This finding is in agreement with a similar study (Hassan *et al.,* 2015) who reported the presence of these active ingredients in *S. siamea* leaves extract. These biologically active metabolites were previously reported to possess antimalarial potentials (Ekasari *et al.,* 2021; Daskum et al., 2020; Javeres *et al.,*2019).

Studies on the medicinal values of plant locally used for the treatment of diseases is well documented in the literature (Javeres *et al.,* 2019; Kharoubia *et al.,* 2008Bako *et al.,* 2005; Adebayo *et al.,* 2003) For example, the antimalarial properties of crude extracts and fractions of medicinal plants has been

ongoing since time immemorial (Alehegn et al., 2020; Misganaw *et al.,* 2019). This has led to the discovery of quinine and artemisinin from Cinchona tree and *Artemisia annua* plants, respectively (Faurant, 2011). Target specific approach such as the Hemin Polymerization pathway is an important method for antimalarial drugs discovery and screening programs from plant sources. Such approaches yield high throughput screening of huge samples in small quantities. Similarly, time, space, energy and resources could be minimized (Basilico *et al*., 1998). The findings of the hemin Polymerization assay reveals a dose dependent inhibition of conversion of hemin to β-hematin. This is in consonance with the results of Olanlokun et al. (2019) and Daskum et al. (2019) who reported a dose dependent inhibition of β-hematin formation by *Diospyros mespiliformis and Mondia whitei*as well as *Senna occidentalis,* respectively. Similarly, Dibua *et al.* (2013) reported that *Alstonia boonei* significantly ($P<0.05$) reduce haemozoin concentration in febrile patients with malaria.
Further assessment of the antimalarial properties of *S. siamea* revealed that synchronized parasite isolates exposed to various doses of crude *S. siamea* leaves extracts also showed suppression of parasitaemia with increased extract dosage. At the lowest concentration of extract, the aqueous extract prevents parasite growth and development by >50% and by 80% at the highest dosage. This substantiate the antimalarial effect of *S. siamea* (Daskum *et al.,* 2020; Tasiam*et al.,* 2020) previously reported. Furthermore, while different IC_{50} values were obtained for each solvent extract, both extracts could be adjudged to possess potent antimalarial activities. This is notwithstanding that extract with $IC_{50}<100$ μg/ml are considered to have some antimalarial potential (Tasiam *et al.,* 2020).
The *in vitro* (HPIA) inhibitory potential and antimalarial activites exerted by crude *S. siamea* extracts may not be unconnected to the synergistic effects of active ingredients identified. Although, medicinal plants are traditionally administered as concoctions, decoctions or topically to treat diseases, activities could be due to one or few of the bioactivie compounds present. It is therefore important to fractionate extracts and identify specific phytochemicals for synthesis of novel antimalarials.

CONCLUSION

Based on the in vitro hemin polymerization activity and antimalarial activities observed, both solvent extracts may be adjudged to possess good antimalarial potentials. These may also contain promising compounds that could be used to develop new antimalarial therapies. Further fraction of bioactive compounds and assessment of *in vivo* antimalarial properties is essential.

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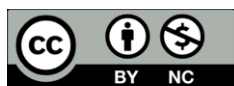

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