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Original article

In Vitro ACTIVITY OF METHANOLIC EXTRACTS OF STEMBARK OF *Anogeisus leiocarpus* (African birch) ON *Plasmodium falciparum*

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ABSTRACT

This study was carried out to evaluate the *in vitro* effect of methanolic extracts of stem bark of *Anogeissuss leiocarpus* (AL) on *Plasmodium falciparum*. Powdered stem bark of *Anogeissus leiocarpus* was subjected to cold maceration using 99.8% methanol. The crude extract was sequentially fractionated using four solvents of increasing polarity. *P. falciparum* field isolate was cultivated *in vitro* using the Trager and Jensen Candle jar method. The plant extracts were tested against the ring staged synchronous *P. falciparum* field isolates by incubation in 96-well micro titre plate for 48hrs. Preliminary Phytochemical screening of the crude methanolic plant extracts revealed the presence of saponins, tannins, flavonoids and cardiac glycosides Results also showed that *Anogeissus leiocapus* extracts had the highest activity when used singly against *P. falciparum* with its crude methanolic extract having IC_{50} of 5.00 µg/ml, ethylacetate fraction with IC_{50} of 5.00µg/ml and benzene fraction with IC_{50} of 9 µg/ml. the other extracts all showed moderate antiplasmodial acitivity with Aqueous fraction having IC_{50} of 17µg/ml and chloroform with IC_{50} of 20 µg/ml. Statistical analysis reveals increasing activity with increasing concentration which shows significant antiplasmodial activity at P value ≤ 0.01. The findings in this studies have shown that the crude methanolic extracts of AL have significant activity on the cultured field isolate of *Plasmodium falciparum*, Therefore the findings in this research will avert the challenges posed by parasitic resistance to the existing antimalarial drugs.

Keywords: Malaria, Antimalarial drugs, *Plasmodium faciparum*, Field isolate, Wet partitioning

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INTRODUCTION

Throughout history, man has suffered from infectious diseases caused by pathogenic microorganisms such as viruses, bacteria, fungi and parasites. Infectious diseases comprise clinically evident illness resulting from the presence and growth of the pathogenic microorganisms in the individual host [1]. A notable example of these infectious diseases, which is a major public health and developmental challenge in Nigeria and many other African countries, is malaria where transmission occurs all year round.

The causative agent of malaria is a parasite in the blood called *Plasmodia.* Four species of this genus causes malaria in humans these are: *P. falciparum, P. vivax, P. malariae* and *P. ovale.* However, *P. knowlesi* associated with long tailed macaque [2]. Malaria contributes substantially to the poor health situation in Africa. About 90% of the world's 216 million cases and 655 000 annual deaths of malaria occur in the sub-Saharan African region [3]. In Nigeria more than 3 million cases are reported annually. A significant proportion of such cases occur in children under the age of five [4]. Treatment of malaria with potent, effective, available and affordable drugs nevertheless, remain crucial to the control and the eventual eradication of the disease in Nigeria and the sub-region as a whole. Many antimalarial chemotherapeutic agents have been used to treat the infection but most of these agents are now not effective due to the widespread of multidrug-resistant malaria parasites [5]. Africa is endowed with numerous plants that have been used traditionally to treat various diseases. And these plants are cheap, readily available and produce less adverse effects compared with conventional drugs. This study focuses *Anogeissus leiocarpus,* also known as african birch of Combretaceae family and Kukunchi in Nupe dialect. The plant was selected through thorough consultations of the people around Bida Local Government in Niger state. This plant is used either singly or in combination locally to cure malaria like ailments such as headaches, high temperatures and seizures

MATERIALS AND METHODS

Collection and authentication of plant materials:

The plant materials were collected in October 2015. The Plants were authenticated in the Department of Biologcal Sciences, Nigerian Defence Academy, Kaduna and the voucher specimens was deposited for future reference with voucher number 1413

Pre-treatment of the plant material:

The plant materials were initially screened for foreign materials such as sand and insects. A stiff brush was used to

clean off the dirt from the roots and stems. Each plant material was washed thoroughly in cold water to remove any trace of unwanted foreign matter.

Drying:

The parts collected were dried under shade for 13 days and when properly dried it was powdered using a grinding machine, then sieved to reduce the size. And then packed in airtight container and stored at room temperature for further analysis.

Plant extraction:

Preparations of crude methanolic extracts of stem bark of *Anogeisus leiocarpus* Maceration:

The powdered plant parts (200g) was weighed using an electronic weighing balance and soaked in 1.2L of 99.8% methanol in a transparent glass bottle. it was shaken intermittently to enhance the extraction process and were left for 48hrs. The filtrate was separated from the residue by sieving with muslin cloth then evaporated under reduced pressure 45(rpm) at 60^0C (in accordance to the boiling point of the solvent i.e. methanol) using a rotary evaporator and then stored in well-closed containers at low temperature (4^0C) in a refrigerator to protect from light and moisture until needed for further analysis [6].

Bioassay guided fractionation of crude extract of *Anogeissus leiocarpus* Stembark

Ten grams (10g) of the crude metanolic extract of the stembark of A.L was first dissolved in 90ml/10mldistilled water and methanol (wet partitioning) in a beaker, then transferred into a separating funnel then Benzene being the solvent with the lowest polarity index was introduced to this suspension in ratio 1:1 as that of water. By using a separating funnel this mixture was separated in to two distinct layers with the aqueous layer which is less dense at the top. The benzene fraction was then separated out. This procedure was also performed on the other polar solvent in order of increasing polarity i.e. chloroform, ethylacetate respectively with polarity index: 2.8>4.1>4.4>9. The fractionation process followed the procedures by [6] with little modification.

Preliminary Phytochemical screening of the crude extract:

The preliminary phytochemical tests were conducted using modified versions of the standard protocols by [7]; [8].

Antimalarial Bioassay

Culture Technique:

The *in vitro Plasmodium falciparum* parasite cultivation was based on the method described by [9]; [10]; [11], called the Candle Jar Method of Culture. Where A white candle was lit and placed within the desiccator containing petri dishes with the *Plasmodium* parasite in a complete medium, and the cover is put on with a stopcock open. When the candle goes out, the stopcock was closed. This is a simple and effective way to produce an atmosphere with low O_2 and high CO_2 content which is optimal for *P. falciparum* growth. The Candle Jar was then incubated at 37 ºC in an incubator.

Malaria Parasite

Field isolates of *Plasmodium falciparum* were used in this antimalarial bioassay because of its accessibility and availability. Isolates were obtained from outpatients at Yusuf Dantsoho memorial Hospital Kaduna.

Collection of blood samples:

Three (3ml) in 0.47ml CPDA-1 (citrate phosphate dextrose adenine) of whole blood (plasmodium infected) samples were collected from patients attending outpatient department of Yusuf Dan Tsoho general hospital, Kaduna in February, 2016. An ethical clearance/permit was obtained from the Ministry of Health, Kaduna state

Screening of Blood Samples
Microscopic identification of *plasmodium falciparum*.

For each collected sample received, thin smear was prepared and stained with giemsa stain to confirm the presence of *Plasmodium* parasite, Parasitemia was determined and growth stage of parasites were observed.

Estimation of the percentage (%) parasitaemia.

An area of stained thin blood film where the erythrocytes are evenly distributed was observed using 100 x objectives (under oil immersion). Approximately 100 erythrocytes in this area were counted. Without moving the slide, the number of infected erythrocytes amongst the 100 erythrocytes was also counted. The slide was moved randomly to adjacent fields and counting was continued as mentioned above. An equivalent of 1,000 erythrocytes was counted. The counting was repeated twice for a total examination of three different parts of the slide, i.e., 3 areas 1000 cells. The mean number of infected RBCs per 1,000 RBCs gives the estimated Percentage (%) parasitaemia in that particular blood sample [12].

Preparation of extract stock solutions:

Crude methanolic extracts and the fractions of. *A. leiocarpus* (stem bark) were screened for antiplasmodial activity against the Laboratory adapted *P. falciparum* isolates. The plant extracts were prepared using culture media RPMI1640 and 0.2% of dimethyl sulphoxide (DMSO) to produce 2mg/ml stock solutions. The stock solutions were sterilised using a 0.4 millipore filter. Subsequently the stock solutions were diluted with culture media to produce six concentrations of the extracts (1, 10, 50, 100, 500, and 1000) µg/ml [9]; [13].

***In vitro* cultivation of *P. falciparum* isolates and susceptibility Testing**:

The assay was performed in triplicate on a 96 wells microtitre plates. The sterile 96 wells tissue culture plate was predosed with 100µl of culture medium containing extracts at various concentrations followed by the addition of 100µl of sub cultured parasite diluted with the O^+ erythrocytes to about 0.5-1% parasitemia. A negative control was maintained with 100µl of *Plasmodium falciparum* culture,100µl of culture medium and positive control was maintained by the addition of a standard drug Arthemeter-lumefantrin at varying concentrations at (1, 10, 50, 100, 500, 1000) µg/ml. The Plates were then incubated in a candle jar in an incubator

at 37^{0}C for 48hrs [9]. After incubation, contents of each well was harvested after carefully removing the culture media which is at the upper layer then Thin blood smears from each well was prepared on a slide and fixed absolute methanol then stained with 10% Giemsa stain at pH 7.3. Number of infected red blood cells was counted with the aid of a compound microscope and the control parasite culture freed from extracts was considered as 100% growth. Antimalarial activity was assessed by parasitaemia determination, determination of the inhibitory concentration as well as IC_{50} (the concentration of extracts that is able to kill 50%.

Parasites growth inhibition per 100 red blood cells was counted in 10 microscopic fields. The control parasite culture freed from extracts was considered as 100% growth. The percentage inhibition per concentration was calculated using the formula:

[(% parasitaemia in control wells – % parasitaemia of test wells)/ (% parasitaemia of the control)] x 100]

The IC_{50} was determined by linear interpolation from the growth inhibition curves (Log of concentration versus percentage inhibition) generated from each parasite-extract interaction [14].

Data Analysis:

with the % parasitaemia decreasing with increase in concentration of the extracts. The data collected was subjected to one-way Analysis of variance (ANOVA) and mean of % parasitaemia of the extracts were compared using Duncan Multiple Range Test (DMRT) at $P \leq 0.01$.

RESULTS

Crude methanol extract of *A. leiocarpus* had a low % yield 10.10%. This is shown in table 1. The preliminary phytochemical analysis of the stembark of *A. leiocarpus* as shown in Table 2 revealed that it contains flavonoids, tannins, saponin and cardiac glycosides. Sequential partitioning of the crude methanolic extract using four solvents of different polarities gave different percentage yields as shown in table 3. The highest percentage yield wad recorded with the aqueous fraction with 50.1% and the lowest % yield in the ethylacetate fraction with 3.8%. The crude methanolic extract of the stem bark of *A. leiocarpus* and the four fractions showed promising inhibitory effect against *P. falciparum*. The crude methanolic extract had the highest effect against the parasite with the lowest concentration 1μg/ml having % Parasitaemia of 53.40. Among the fractions of *A. leiocarpus* stembark Benzene had the highest effect and the lowest was found to be the aqueous fraction this is all shown in Table 4. It was also observed that the antiplasmodial activity is dose dependent.

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Table 1: % Yield of Crude methanolic extract of *A.l*

Plant species A.L	Initial Weight(g)	Final weight(g)	% Yield
	200	10.2	5.10

Keys: A.L= *Anogeissus leocarpus*

Table 2: Percentage yields of fractions of methanolic Stembark of *A. leiocarpus*

Plant species	Solvent, (PI)	Initial Weight(g)	Final weight(g)	% Yield
Anogeissus leiocarpus	Benzene, (2.8)	10	2.96	29.60
	Chloroform, (4.1)	10	1.10	10.10
	Ethylacetate, (4.4)	10	0.38	03.80
	Aqueous, (9.0)	10	5.10	50.10

Key: PI= Polarity index

Table 3: Phytochemical components detected in *Anogeisus leiocarpus stem bark* extract

	A. leiocarpus (stem bark)
Tannins	+ +
Saponins	+ +
Anthraquinones	_
Flavonoids	+ + +
Alkaloids	_
Cardiac glycoside	+
Steroids	+

Key: +++: Highly present. ++: moderately present, +: Presence in trace,_:Absent

Table 4. Antiplasmodial activity of Crude methanolic extract of stem bark of *A. leiocapus* and

	Concentration in µg/ml					
	1000	500	100	50	10	1
Plant Extracts	% Mean Parasite Growth ±SD					
Crude M AL	2.6 ± 0.2^{b}	7.40 ± 0.4^{b}	15.79 ± 0.2^{b}	22.10 ± 3.7^{b}	36.50 ± 0.7^{b}	53.40 ± 1.5^{d}
Benzene fraction	6.20 ± 0.2^{c}	11.40 ± 4.0^{ab}	31.00 ± 2.0^{bc}	38.20 ± 5.0^{b}	57.36 ± 1.8^{c}	66.30 ± 2.1^{b}
Chloroform frac	10.62 ± 0.3^{d}	19.70 ± 0.3^{ab}	23.60 ± 0.2^{a}	64.14 ± 1.0^{a}	77.93 ± 1.6^{a}	89.00 ± 4.0^{a}
Ethyacetate frac	19.54 ± 0.2^{a}	24.10 ± 0.5^{a}	38.30 ± 0.9^{c}	43.60 ± 0.2^{b}	49.80 ± 1.0^{d}	65.17 ± 2.1^{c}
Aqueous fraction	21.30 ± 2.0^{a}	24.71 ± 0.1^{a}	24.56 ± 1.4^{a}	54.60 ± 0.9^{a}	71.29 ± 4.4^{a}	82.00 ± 3.8^{a}

the various fractions: Benzene, Chloroform, Ethylacetate and Aqueous

Keys: Crude M AL= Crude methanolic extract of *A. leiocarpus*. % mean parasite values followed by the same superscript in the same column are not significantly different at P<0.01. (Duncan Multiple Range Test).

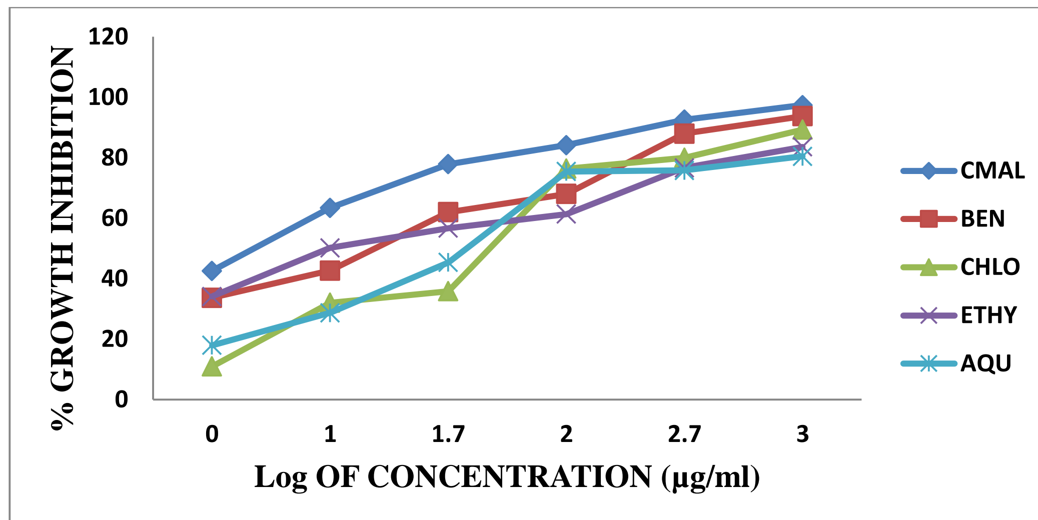

Figure 1. The percentage inhibition of the extracts of *Anogeisus leiocarpus* stembark showing dose-dependent antiplasmodial activity.

DISCUSSION

The successful extraction of bioactive compounds from plants, according to Parekh *et al* (2005) is largely dependent on the type of solvent used in the extraction procedure. Effective extraction from the dried plant material was achieved using alcohol specifically methanol a slightly non-polar solvent used for herbal active components as this enhances the isolation of both polar and non-polar secondary metabolites [15]; [16]. *A. leiocarpus* had the highest % yield in the aqueous fraction following slightly by a non-polar solvent i.e. the benzene fraction with 29%, this is consistent with [17], who maintains that these observations can be explained due to the polarity of the compounds extracted. Phytochemical screens determine the overall chemical "fingerprint" or "chemical profile" of a plant extract [18]. Phytochemical test for the seven compounds revealed that the stem bark of *A. Leiocarpus* contains Tanins, Saponins, flavonoids,cardiac glycosides but devoid of Anthraquinones and Alkaloids and this agreess with the findings of [19]. Previous studies have shown that the composition of Phytochemicals present in the plant has direct correlation with its pharmacological activity [18]. Thus the antiplasmodial properties of these p extracts may be attributed to these phytochemicals identified.

In this study five extracts were evaluated for antiplasmodial activity *in vitro* (go to results). Among the five extracts, the lowest IC_{50} was observed with the crude methanolic extract of *A. leiocarpus* with

IC_{50} of 5.0 µg/ml followed by the Ethylacetate fraction with IC_{50} of 7µg/ml. Benzene fraction had IC_{50} of 9 µg/ml then Chloroform and Aqueous fraction had 17µg/ml and 20µg/ml respectively. Literatures indicated that plant extracts with *in vitro* antiplasmodial activity with IC_{50} less than 10µg/ml as highly active. Those with IC_{50} between 10µg/ml and 50µg/ml are classified as moderately active while the extracts with IC_{50} greater than 50 as inactive [20]; [21]. Base on this classification the crude methanolic extracts, the benzene fraction and the ethylacetate fractions are all highly active against the parasite. The results is in line with the results of [22] who reported a high antimalarial activity when methanol extract of leaves of the plant was used against *Plasmodium falciparum* strain FCB1 with IC_{50} of 2.60µg/ml. Although this research was carefully prepared and has achieved its aim, there were some unavoidable limitations. First, the RPMI 1640 media was very susceptible to contaminations and even while they were stored in aliquots some had to be discarded due to contaminations and secondly, artefacts were seen on the microscopic slides when determining the parasitemia. Further studies such as the in vivo antiplasmodial assay in animal models should be conducted to investigate how liver metabolism affects the efficacy of the plant extracts.

CONCLUSION

The current studies have shown that the crude methanolic and fractions of stem bark of *A. leiocarpus* had significant activity on the cultured field isolate of *Plasmodium falciparum*. Thus, the results validate the traditional use of this plant as treatment for malaria. The findings will contribute to the ongoing efforts to eliminate malaria. This will be achieved when the bioactive compounds in these extracts are investigated, thus leading to the development of a novel antimalarial drug.

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