

IN VITRO ACTIVITY OF METHANOL EXTRACTS OF ROOT OF SARCOCEPHALUS LATIFOLIUS (AFRICAN PEACH) ON PLASMODIUM FALCIPARUM

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ABSTRACT

This study was carried out to evaluate the *in vitro* effect of crude methanol extracts of root of *Sarcocephalus latifolius* (SL) on *Plasmodium falciparum*. Powdered root of *Sarcocephalus latifolius* 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 48 h. Preliminary Phytochemical screening of the crude methanol plant extracts revealed the presence of Saponins, Tannins, Flavonoids, Alkaloids, Anthraquinone and cardiac glycosides. Results also showed that *Sarcocephalus latifolius* extracts have moderate activity when against *P. falciparum* with its crude methanol extract having IC₅₀ of 14.78 µg/mL, chloroform fraction with 19.95 µg/mL, Benzene fraction with IC₅₀ of 33.00 µg/mL and ethylacetate fraction with IC₅₀ of 49 µg/mL. The Aqueous fraction had the lowest antiplasmodial activity with IC₅₀ of 72 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 methanol extracts of SL and its fractions have moderate activity on the cultured field isolate of *Plasmodium falciparum*. Therefore the findings in this research will serve as a stepping stone to avert some of the challenges posed by parasite resistance to the existing antimalarial drugs.

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

INTRODUCTION

The world continues to be plagued from infectious diseases caused by pathogenic microorganisms such as bacteria, viruses, fungi and parasites. Throughout history, man has suffered from infectious diseases caused by pathogenic microorganisms such as viruses, bacteria, fungi and parasites. Infectious diseases are clinical manifestation resulting from the presence and proliferation of the pathogenic organisms in a susceptible host (Antabe & Ziegler 2020). 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 it is endemic and 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* (Lee *et al.*, 2011). According to the latest World malaria report, there were

249 million cases of malaria in 2022 compared to 244 million cases in 2021. The estimated number of malaria deaths stood at 608 000 in 2022 compared to 610 000 in 2021 (WHO, 2023). African Region continues to carry a disproportionately high share of the global malaria burden. In 2022 the Region was home to about 94% of all malaria cases and 95% of deaths. Children under 5 years of age accounted for about 78% of all malaria deaths in the Region (Haileselassie *et al.*, 2023).

Four African countries accounted for just over half of all malaria deaths worldwide: Nigeria (26.8%), the Democratic Republic of the Congo (12.3%), Uganda (5.1%) and Mozambique (4.2%) (Haileselassie *et al.*, 2023). 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 used as treatment regimen has met with a lot of drawbacks such as resistance, cost and availability (Rasmussen *et al.*, 2022). Africa, Nigeria inclusive is endowed with numerous plants that are used traditionally to treat various ailments. These plants are readily available in our environment and produce less adverse effects compared with conventional drugs. This study focuses *Sarcocephalus latifolius*, also known as African peach of *Rubiaceae* family and Gbashi 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, fever and malaise.

MATERIALS AND METHODS

Ethical Clearance

Approval to carry out the study was obtained from the ethical committee of Kaduna State Ministry of Health.

Collection and Authentication of plant materials:

The plant materials were collected in October, 2015. The Plants were authenticated in the Department of Biological 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 14 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 methanol extracts of Root of *Sarcocephallus latifolius* Maceration:

The powdered plant parts (200 g) was weighed using an electronic weighing balance and soaked in 1.2 L 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°C (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°C) in a refrigerator to protect from light and moisture until needed for further analysis (Sutherson *et al.*, 2007).

Bioassay guided fractionation of crude extract of *Sarcocephallus latifolius* Root

Ten grams (10 g) of the crude methanol extract of the root of SL was first dissolved in 90 mL/10 mL distilled 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 into 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 Sutherson *et al.*, 2007, with little modification.

Preliminary Phytochemical screening of the crude extract:

The preliminary phytochemical tests were conducted using modified versions of the standard protocols by Njoku & Obi (2009); Nobakht *et al.*, (2010).

ANTIMALARIAL BIOASSAY

Culture Technique:

The *in vitro Plasmodium falciparum* parasite cultivation was based on the method described by Trager and Jensen (1976), (1977), 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 O₂ and high CO₂ content which is optimal for *P. falciparum* growth. The Candle Jar was then incubated at 37 °C 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 (3) mL in 0.47 mL 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.

Molecular Characterization

200 µL of whole blood was added into a 1.5 mL Ependoff tubes. Four hundred (400) µL of lysis buffer and 10 µL of proteinase K was added and the tube was placed on a heating block which was set at 60°C and allowed to heat for 1 hr. Four hundred (400) µL of phenol: chloroform (1:1) was added to the lysate and vortexed briefly for 2 min. Contents were centrifuged at 10000 rpm for 10 min to separate the phases. The upper layer was carefully removed with a micro-pipette and transferred into a 1.5 mL tube. Four hundred (400) µL of chloroform was carefully added and vortexed briefly before spinning at 10000 rpm for 10 min. The upper clear layer was carefully transferred to another 1.5 mL tube without extracting the interface. Equal volume of 100% ethanol and 20 µL of 3 M sodium acetate was added and mixed carefully by gently shaking the tubes before incubating at 20°C for 24 h. After incubation, the tube was centrifuged at maximum speed for about 20 min, the ethanol portion was removed and 400 µL of 70 % ethanol was added and centrifuged at maximum speed for 5 min at 4°C. The resultant DNA was dried out by leaving the tube opened for 3-10 min.

PCR primers were purchased from Integrated DNA Technologies, Inc. (IDT), Coralville, Iowa, USA. Primers [P_{f1} (5' GGA ATC TTA TTG CTA ACA 3') P_{f2} (5'AAT GAA GAG CTG TGT ATC3')] that are species specific for *Plasmodium falciparum* (P_{f1} and P_{f2}) were used for the amplification to confirm the *P. falciparum* status. Amplification was carried out in a DNA thermal cycler GeneAmp® PCR System 9700 Applied Biosystems®. Two (2) µL of the extracted DNA was suspended in a 20 µL (final volume) reaction Accupower® Hotstart PCR premix containing 1 unit of Hot-start DNA polymerase, 1× PCR buffer, 250 µM of each dNTPs and 1 µL P_{f1}/P_{f2} (P_f PCR). The amplification step included 1 min "denaturation" at 94°C, 1 min "annealing" step at 56°C *P. falciparum* PCR for 30 cycles and lastly, a 1 min "elongation" step at 72°C. The thermal cycler temperature was maintained at 4°C after the final cycle, until the samples were removed (Berry *et al.*, 2008).

Amplified PCR products were detected by gel electrophoresis. A standard 1.5% agarose gel was prepared by dissolving 1.5 g of agarose powder in 100 mL of Tris acetate EDTA (stained using a solution of ethidium bromide (0.5 µg/mL). Eight (8) µL of each of the samples was loaded and subjected to electrophoresis for 35 min at a voltage of 100 V and current of 40 mA. The gel was visualized with ultraviolet light and photographed using Gel-doc system. The band sizes for the PCR products were observed. Blood sample with mono-infection of *Plasmodium falciparum* after

both microscopic and molecular screening were used for the *in vitro* cultivation (WHO, 2016).

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 (Koudouvo *et al.*, 2011).

Preparation of extract stock solutions: Crude methanolic extracts and the fractions of *S. latifolius* Root were screened for antiparasmodial 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 2 mg/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 (Trager & Jensen 1977; Koudouvo *et al.*, 2011).

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 pre-dosed with 100 µL of culture medium containing extracts at various concentrations followed by the addition of 100 µL of sub-cultured parasite diluted with the O⁺ erythrocytes to about 0.5-1% parasitemia. A negative control was maintained with 100 µL of *Plasmodium falciparum* culture, 100 µL of culture medium and positive control was maintained by the addition of a standard drug Artemeter-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°C for 48 hr. 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 IC₅₀ (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:

$$\left[\frac{(\% \text{ parasitaemia in control wells} - \% \text{ parasitaemia of test wells})}{(\% \text{ parasitaemia of the control})} \right] \times 100$$

The IC₅₀ was determined by linear interpolation from the growth inhibition curves (Log of concentration versus percentage inhibition) generated from each parasite-extract interaction (Mustofa *et al.*, 2007).

Data Analysis:

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 ≤ 0.01.

RESULTS

Crude methanol extract of *S. latifolius* had a % yield 10.81%. This is shown in Table 1. Sequential partitioning of the crude methanol extract using four solvents of different polarities gave different percentage yields as shown in Table 2. The highest percentage yield was recorded with the aqueous fraction with 48.30% and the lowest % yield in the Benzene fraction with 0.8%. The preliminary phytochemical analysis of the root of *S. latifolius* as shown in Table 3 revealed that it contains flavonoids, tannins, Alkaloids, anthraquinone, saponin and cardiac glycosides. The crude methanolic extract of the Root of *S. latifolius* 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 1 µg/mL having % Parasitaemia of 77.28. Among the fractions of *S. latifolius* root Chloroform fraction 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 antiparasmodial activity is dose dependent with the % parasitaemia decreasing with increase in concentration of the extracts.

Table 1: Percentage Yield of Crude Methanol Extract of *S. latifolius*

Plant species	Initial Weight (g)	Final weight (g)	% Yield
S.L	200	21.62	10.81%

Keys: S.L= *Sarcocephallus latifolius*

Table 2: Percentage yields of fractions of methanol Stem bark of *S. latifolius*

Plant species	Solvent, (PI)	Initial Weight(g)	Final weight(g)	% Yield
<i>Sarcocephallus latifolius</i>	Benzene, (2.8)	10	0.81	08.10
	Chloroform, (4.1)	10	1.35	13.50
	Ethylacetate, (4.4)	10	2.57	25.70
	Aqueous, (9.0)	10	4.83	48.30

Key: PI= Polarity index

Table 3: Phytochemical components detected in *S. latifolius* root extract

<i>S. latifolius</i> (Root)	
Tannins	+++
Saponins	+
Anthraquinones	++
Flavonoids	++
Alkaloids	+
Cardiac glycoside	++
Steroids	-

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

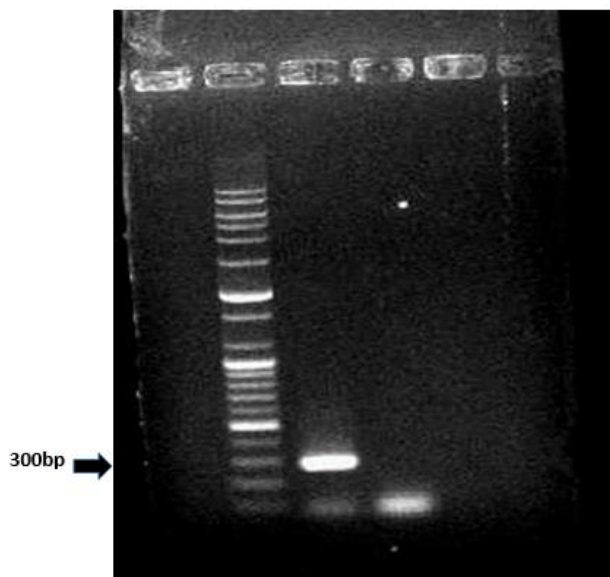


Plate 1. PCR Electrophotograph of specie-specific detection of *P. falciparum*. (Lane 1, 1kb ladder 2Amp. *P. falciparum*, Negative control: pFr, primer fronts).

Table 4. Antiplasmodial activity of *S. latifolius* Root Extracts

Plant Extracts	Concentration in $\mu\text{g/mL}$					
	1000	500	100	50	10	1
	% Mean Parasite Growth \pmSD					
Crude M SL	6.74 \pm 0.2 ^b	13.10 \pm 0.4 ^a	24.79 \pm 0.2 ^a	36.10 \pm 1.6 ^a	63.50 \pm 0.5 ^b	77.28 \pm 1.5 ^d
Benzene fraction	8.24 \pm 0.2 ^d	18.26 \pm 4.0 ^{bd}	42.00 \pm 2.0 ^{bc}	58.40 \pm 5.0 ^b	61.30 \pm 1.8 ^b	82.24 \pm 2.1 ^b
Chloroform frac	7.02 \pm 0.3 ^d	16.70 \pm 0.3 ^b	28.60 \pm 0.2 ^b	45.14 \pm 1.0 ^a	58.13 \pm 1.6 ^a	79.46 \pm 4.0 ^a
Ethylacetate frac	12.54 \pm 0.2 ^a	26.14 \pm 0.5 ^{bd}	45.43 \pm 0.9 ^c	60.60 \pm 0.2 ^c	71.31 \pm 1.0 ^c	85.32 \pm 2.1 ^c
Aqueous fraction	14.0.1 \pm 2.0 ^a	31.21 \pm 0.1 ^c	58.16 \pm 1.4 ^c	62.40 \pm 0.9 ^c	76.28 \pm 4.4 ^c	85.00 \pm 1.8 ^a

Keys: Crude M SL= Crude Methanol extract of *S. latifolius*. % mean parasite values followed by the same superscript in the same column are not significantly different at $P < 0.01$. (Duncan Multiple Range Test)

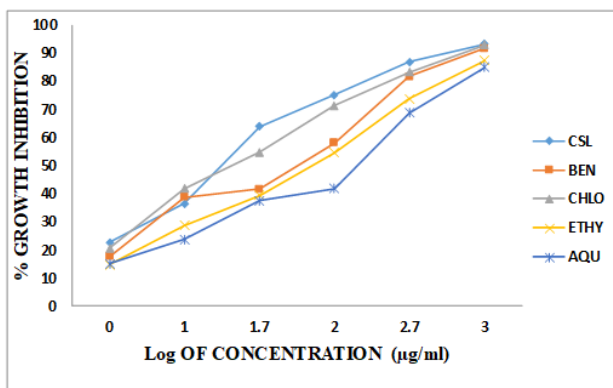


Fig 1. The percentage inhibition of the extracts of *Sarcoccephallus latifolius* Root showing dose-dependent antiplasmodial activity

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 Shuaibu *et al.*, (2008); Jordana *et al.*, (2010). *S. latifolius* had the highest % yield in the aqueous fraction followed by the ethyl acetate fraction with 25%, this is consistent with Parekh *et al.* (2005) 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. Phytochemical test for the seven compounds revealed that the Root of *S. Latifolius* contains Tanins, Anthraquinones, Alkaloids, Saponins, flavonoids, cardiac glycosides but devoid of steroids this

agrees with the findings of (Iheagwam *et al.*, 2020). Previous studies have shown that the composition of Phytochemicals present in the plant has direct correlation with its pharmacological activity (Bandaranayake *et al.*, 2008). Thus the antiplasmodial properties of these plant extracts may be attributed to these phytochemicals identified.

PCR based assay has been developed for the detection and identification of malaria parasite to overcome some of the limitations of microscopy and has proven to be more specific and sensitive (Mbanefo and Kumar, 2020). In this study, PCR was carried out on the blood containing the cultured *P. falciparum* using specific primers and a band size of 300bp was seen thus confirming the presence of the parasite.

In this study five extracts were evaluated for antiplasmodial activity *in vitro* (go to results). Among the five extracts, the lowest IC₅₀ was observed with the crude methanol extract of *S. latifolius* with IC₅₀ of 14.78 µg/mL followed by the Chloroform fraction with IC₅₀ of 19.95 µg/mL. Benzene fraction had IC₅₀ of 33 µg/mL then ethylacetate and Aqueous fraction had 49 µg/mL and 72 µg/mL respectively. Literatures indicate that plant extracts with *in vitro* antiplasmodial activity with IC₅₀ less than 10µg/ml as Highly active. Those with IC₅₀ between 10 µg/mL and 50 µg/mL are classified as moderately active while the extracts with IC₅₀ greater than 50 as inactive (Razamani *et al.*, 2010; Omoregie & Sisodia, 2012). Base on this classification the crude methanol extract, the benzene Chloroform and the ethylacetate fractions are moderately active against the parasite. The results is in line with the results of Udofia *et al.*, (2019) who reported a moderate activity and dose dependent toxicity antimalarial when methanol extract of the root of the plant used against mouse infected with *Plasmodium berghei* with 400 mg/kg reducing parasitemia significantly. 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 methanol and fractions of root of *S. latifolius* had moderate 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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