Assessment of Phytochemical Constituents and *In Vivo* Antimalarial Activity of Methanol Extract and Solvent Fractions of *Phyllanthus amarus* Schum. and Thonn.

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Abstract

Background: The scourge of malaria epidemic is still felt in sub-Saharan Africa, especially in Nigeria, despite the dedicated efforts directed toward its eradication. Therefore, the search for new, affordable and more potent antimalarial drugs from plants, especially, has become frontline in recent times. Traditional plants like *Phyllanthus amarus* are said to possess numerous phytochemicals responsible for their notable biological activities. **Objectives:** This study evaluates the phytochemical constituents, acute oral toxicity and *in vivo* antiplasmodial activity of methanol extract and its solvent fractions using documented methods. **Results:** Results from our study highlight the nontoxicity of the methanol extract and fractions. Qualitative phytochemical analysis of the methanol extract indicated the presence of a wide array of phytochemicals, while quantitative analysis revealed alkaloids as the most abundant phytochemical in both the methanol extract (3.82±0.53%) and the chloroform fraction (2.96±0.48%). The chloroform fraction showed the greatest parasite suppression in early (87%) and established (96%) infections. However, the methanol extract showed greater prophylactic activity (93%) in comparison with the chloroform fraction (66%). Parasite suppression by the chloroform fraction in tissues showed a dose-dependent increase in suppression. Also, its amelioration on parasite-altered haematological indices was significant. **Conclusion:** The chloroform fraction of *P. amarus* methanol extract possesses antimalarial phytochemicals with a promising source of lead compounds for drug discovery. So, the compounds should be identified for study.

Keywords: Antimalarial drug; *Phyllanthus amarus*; Alkaloids; Methanol extract; Chloroform fraction; Prophylactic activity, Suppression

INTRODUCTION

Malaria occurs throughout most of the tropical regions of the world, with *Plasmodium falciparum* causing the largest burden of disease (WHO, 2022). Globally, the World Health Organization recorded approximately 247 million malaria cases in 2021, which was an increase from the 241million cases recorded in the previous year. The WHO African region accounted for 96% of these cases with Nigeria having the highest number of cases (27%) in the world (WHO, 2022).

Over the years development of resistant strains of malaria has placed a barrier in effective treatments. While, most medications used in malarial treatments are targeted at parasitic forms in the blood (Shingadia, 2014), malaria chemotherapeutics are divided into pre-erythrocytic (sporozoite and liver-stage), blood-stage, and transmission-blocking agents (Vuk et al. 2008; Arama and Troye-Blomberg 2014). Artemisinin combination therapy (ACT), the WHO-recommended first-line regimen for the treatment of malaria in sub-Saharan Africa, is targeted at parasitic forms in blood (Dondorp et al. 2010). However, the growing resistance of parasites to commonly used WHO recommended drugs has continue to threaten the success of chemotherapy. Therefore, the need for alternative sources has become imperative. Plant source is largely being considered by researchers because of the possibility of developing phytocompounds into drugs. One of such plants that have shown great promise based on traditional use is *Phyllanthus amarus*.

Phyllanthus (*P.*) *amarus* Schumach. & Thonn is a plant commonly used in traditional medicine for it's hepatoprotective, anti-diabetic, antihypertensive, analgesic, anti-inflammatory, and antimicrobial properties (Adeneye et al. 2006). Decoction of the plant parts has been used in traditonal medicine for the treatment of dysentery diarrhoea, jaundice, hepatitis, kidney stones, gall bladder infections, menstrual or uterine cramps (Srividya and Perival, 1995; Patel et al., 2011). The Plant contains constituents that are bioactive and thus, are used in the treatment of many human diseases. These activities of *P. amarus* have been linked to the presence of a vast number of phytochemicals in its extracts. Alkaloids , flavonoids, phenols, saponins, terpenes, glycosides, anthraquinones, carbohydrate and sterols have been confirmed (Adegoke et al. 2012; Dhongade and Chandewar, 2012; Simon et al. 2014; Egbon et al. 2017; Bankole et al. 2019; Oshomoh and Uzama-Avenbuan, 2020; Saranraj and Sivasakthivelan, 2012) to be present in the plant.

The antiplasmodial activity of *Phyllanthus amarus* has also been reported (Ajala et al. 2011; Onyesom et al. 2015; 2019; Uzuegbu et al. 2020). Hence, studying the antiplasmodial potential of the extracts of *P. amarus* based on its many traditional uses could provide alternative source of medicine for malaria therapy. This study, therefore, assesses the antiplasmodial activity and phytochemical constituents of the methanol extract of *P. amarus* and its solvent fractions (chloroform, aqueous, ethanolic), intending to identify the phytochemicals responsible for its activity and the best solvent for extracting these active ingredients.

MATERIALS AND METHODS

Extraction of plant materials

Fresh, full grown whole plants of *P. amarus* were collected from their natural environment in Abraka, Ethiope East Local Government Area of Delta State, Nigeria, and identified by a taxonomist at the Forestry Research Institute of Nigeria, Ibadan, Oyo State, Nigeria (voucher No. FHI 109728) by comparing it to samples at the Institute.

Whole plants were washed and air dried for two weeks at room temperature (28 ^{o}C -32 ^{o}C) to a constant weight. Dried whole plants were powdered using a laboratory blender (Kenwood, Japan). Nine hundred grams (900 g) of the dried, powdered plant was extracted by maceration in 6 L of methanol (98% v/v) at room temperature for 2 days to obtain crude methanol extract which was filtered. The residue was then, reconstituted in 4 L of methanol (98% v/v) and the extraction process repeated. The filtrates were concentrated under reduced pressure using a rotary evaporator to obtain 98 g of the crude methanol extract (ME). Fractions from the methanol extract were prepared using the methods previously described (Ifeoma et al. 2013). About 33 g of methanol extract was distributed between water and chloroform (4 x 600 mL) using a separation funnel. The lower suspension was concentrated to dryness under vacuum by rotary evaporation at 40 ^{o}C. The semi-liquid mixture obtained was the chloroform fraction (9.09% w/w). The upper layer was concentrated and extracted with ethanol (96% v/v) and evaporated to dryness to give the ethanol fraction 18.18%). The remaining residue after ethanol extraction was evaporated to dryness to give the aqueous fraction (54.55%).

Phytochemical screening

The presence of alkaloids (Mayer's test), flavonoids (lead acetate test), terpenes (Salkowski s test), anthraquinones (Borntrager's test), phenols (ferric chloride test), saponins, tannins, and resins were detected according to methods stated by Santhi and Segottuvel (2016). Detection of the presence of carbohydrates (Molish's test), glycosides (Legal's test), and volatile oils were carried out by methods previously described by Banu and Catharine (2015), while phlobotanins and steroids were detected according to Ajiboye et al. (2013) and reducing sugars (Fehling's test) according to Usunobun and Ngozi (2016).

Quantitative Phytochemical analysis

Estimation of total phenols

The sample was defatted and boiled with 50 mL of ether for 15 min. A mixture was then prepared by adding 2 mL of ammonium hydroxide solution, 10 mL of distilled water, 5 mL of conc. amyl alcohol, and 5 mL of extract/fractions. The sample was left to stand for 30 min. Thereafter, absorbance was read using a spectrophotometer at 505 nm (Awomukwu et al. 2014)

Estimation of total flavonoids

The method of estimating total flavonoids was based on the formation of the flavonoidaluminum complex which is absorbed at 415 nm. So, 100 µL of the extract in methanol (10 mg/mL) was immersed in 100 µL of 20 % aluminum trichloride in methanol, a drop of acetic acid, and 5mL of methanol. The absorption at 415 nm was read after 40 min. For blank samples, 100 mL of extract was added to a drop of acetic acid and then, diluted to 5mL with methanol. The absorption of standard solution (0.5 mg/mL) in methanol was measured under the same conditions (Kumaran and Karunakaran, 2016).

Estimation of total alkaloids

About 200 mL of 20 % acetate was added to 100 µL of individual fractions in test tubes and left to stand for 4 h. Conc. ammonium hydroxide was then, added in drops until a precipitate was complete. The precipitate was then collected and weighed (Obadoni and Ochuko, 2001). The percentage of total alkaloid content was calculated as follows:

$$(\text{Weight of residue}/\text{Weight of sample taken}) \times 100$$

Estimation of total tannins

Tannins content of *P. amarus* was estimated by the method of Siddhuraj and Manian (2007). About 500 µL of the extracts were taken in a test tube separately and treated with 100 mg of polyvinyl polypyrrolidone and 500 µL of distilled water. The solution was then, incubated at 4 °C for 4 h and thereafter, centrifuged at 5 000 r/min for 5 min. Then, 20 µL of the supernatant was taken and the phenolic content of the supernatant was measured at 725 nm and expressed as the content of free phenolic on a dry matter basis. Total tannins contained in the extract was calculated as follows:

Total tannins (mg GAE/extract) = Total phenolic (mg GAE/extract) – Free phenolic (mg GAE/extract)

Estimation of total saponins

The estimation of total saponins content was performed by the method according to Goel et al (2012). About 10 mg of extract and individual fractions were dissolved in 5 mL of 80% aqueous methanol. Then, 50 µL of the mixture was added to different test tubes containing 0.25 ml of vanillin reagent. Test tubes were placed in ice-cold water bath and 2.5 ml of 72 % sulphuric acid was added slowly to the solution. Test tubes were left to stand for 3 min and then, heated at 60^0C for 10 min. Absorbance at 544nm was read after cooling using a spectrophotometer.

Estimation of total glycosides

Glycoside content in the sample was evaluated using Buljet's reagent as described by El-Olemy et al. (1994). About 100 µL of individual fractions were collected into test tubes. Freshly prepared Buljet's reagent (containing 95ml aqueous picric acid + 5mL 10% aqueous NaOH) was then added to extracts. The difference between the intensity of colours of the experimental and blank (distilled water and Buljet's reagent) samples gives the absorbance and is proportional to the concentration of the glycosides.

Experimental animals

Adult Swiss mice (BALB/c albino strain), about eight weeks old, weighing between 24-28 g were obtained from Laboratory Animal Centre, LAC, Faculty of Basic Medical Sciences, FBMS, Delta State University, DELSU, Abraka, Nigeria. The experimental mice were

maintained in strict compliance with guidelines approved by the Research and Bioethics Committee, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.

Acute oral toxicity

The animals were divided into the control (0 mg/kg) and six treated groups, treated with methanol extract and its solvent fractions (chloroform, ethanol, and aqueous fractions). The treated groups consisted of five animals for each dose of 0, 10, 100, 1000, 2000, 3000, and 5000 mg/kg. Acute oral toxicity was determined according to methods described by the OECD (Organization for Economic Co-operation and Development), with some modifications. Mice were contained in stainless steel wire mesh cages in an experimental animal room at room temperature (23 ± 3°C) and 50-60% humidity, for 5 days prior treatment. The animal room was sustained at a 12-hour light/ dark cycle to allow adaptation of experimental animals to the laboratory environmental conditions. The mice were administered with various doses of extracts. Food was provided after 1 h of dosing. The mice were observed carefully for any signs of toxicity in the first four hours after the treatment period, and daily thereafter for a period of 14 days. Observations were recorded methodically; individual records were maintained for each mouse.

Parasite inoculation

Donor mice, already inoculated with *P. berghei* parasites (Strain NK65), were obtained from the Department of Parasitology, Nigerian Institute of Medical Research, NIMR, Yaba, Lagos State, Nigeria. The experimental mice were infected by obtaining parasitized blood from the cut tail tip of the infected (donor) mice. The inoculum was prepared using phosphate-buffered saline (PBS). Then, 0.1 mL of infected blood was diluted in 0.9 mL of PBS, pH 7.2.

Antiplasmodial activity of *P. amarus* methanol extract and its solvent fractions (chloroform, aqueous, ethanolic) in early infection.

This was carried out according to methods previously described by Kalra et al. (2006) with some modifications. Seventy (70) mice were selected and arranged into groups of five mice each, based on their mean weights. On the first day, within 4 h after inoculation of mice with the parasite, treatment of all groups began. Groups treated with standard drug Lonart®DS and distilled water, served as positive (Group 1) and negative (Group 2) controls, respectively.

Test groups were, therefore, separated into 12 groups treated with varying doses (100, 300, and 500 mg/kg) of the methanol extract and (50, 75, and 100 mg/kg) for each solvent fraction.

The *P. amarus* methanol extract, solvent fractions and standard drug (Lonart®DS) doses were administered once daily as designed, using intragastric cannula for a period of four days. Parasitaemia was assessed on day 4 by thick blood smears made by collecting blood from the cut tail tip of only the infected mice and stained with Giemsa stain which was later viewed under the microscope (TH- 9845, Serico, China) at ×40 magnification. Percentage parasitaemia and chemosuppression were calculated using the following formulae:

% Parasitaemia = (Number of parasitized RBCs / Total number of RBCs) ×100

% Chemosuppression = [(Mean parasitaemia of negative control – Mean parasitaemia of treated group) / Mean parasitaemia of negative control) × 100

Based highest activity of the chlroform fraction in early infection, only this fraction was then, selected for further analysis along with the methanol extract.

Antiplasmodial activity of *P. amarus* methanol extract and its chloroform extract in established infection.

Antiplasmodial activity in established infection was evaluated using methods previously described by Onyesom et al. (2015). After 72 h of inoculation, blood smears were prepared to determine pre-treatment parasitaemia and treatment commenced. The mice were thereafter, separated into eight (8) groups of five, based on the mean parasitaemia. As stated above, groups treated with the standard drug and distilled water remain our positive (Group 1) and negative (Group 2) controls respectively. Test groups were, therefore, separated into 6 groups treated with varying doses (100, 300, and 500 mg/kg) of methanol extract and (50, 75, and, 100 mg/kg) chloroform fraction.

Parasitaemia was assessed at Day 0, 3, 6, and 9 by thick blood smears made by collecting blood from the cut tail tip of only the infected mice and stained with Giemsa stain which was later viewed under the microscope (TH- 9845, Serico, China) at ×40 magnification. Percentage parasitaemia was then calculated as already stated above.

Haematological indices induced by *P. amarus* chloroform fraction of the methanol extract in established infection

Twenty-four (24) h after the last treatment, control groups, and groups treated with chloroform fraction were euthanatized. Then, 1 mL of blood was then collected from each animal by cardiac puncture with a sterile syringe and needle into EDTA treated screw-cap sample bottles. Blood samples collected were used for haematological analysis.

Within 24 h of sample collection, full blood counts which include packed cell volume (PCV), red blood cell (RBC), haemoglobin (Hb), white blood cells (WBC), platelet count, differential white blood cell (monocytes, neutrophils) and red cell indices including mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) were estimated using the Sysmex® Automated Hematology Analyzer KX-2IN, Sysmex Corporation, Kobe, Japan

Prophylactic antiplasmodial activity of *P. amarus* methanol extract and its chloroform fraction

Prophylactic antiplasmodial activity of methanol extract and the chloroform fraction was carried out by methods stated above with some modifications. The mice were separated into eight (8) groups of five. Grouping of mice was done according to methods stated above and received the same dose of extract or fraction once daily for three consecutive days. On day 4, the mice were inoculated with *Plasmodium berghei*. After 72 h of inoculation, thin blood films of each mouse were made and parasitaemia was, hence, calculated.

Parasite suppression in tissues by *P. amarus* chloroform fraction in established malarial infection

Animals were grouped into positive control, negative control, and chloroform fraction-treated groups as stated above. Then, 24 h after the last treatment, the mice were fasted overnight and euthanized. Thereafter, kidney, brain, liver, heart, pancreas, testis, epididymis, ovary, uterus, and blood specimens were collected and processed as required for analysis. About, 0.5 g of tissue samples were homogenized individually with phosphate buffer of 7.2 pH to make a smear on clean microscopic slides. Dried smears were then stained with Giemsa and read with a microscope. Parasitaemia was then, read and calculated using the aforementioned formula.

Statistical analysis

Data were analyzed with ANOVA using GraphPad Prism (GraphPad Software Inc, LLC, San Diego CA, USA, version 6). Results were expressed as Mean ± SD. Values were considered significant at $p<0.05$

RESULTS

Results from present study highlights the phytochemical analysis and antiplasmodial activity of the methanol extract and solvent fractions of *Phyllanthus amarus* Schum and Thonn.

Qualitative and quantitative analysis of phytochemicals of *P. amarus*

Results in Table 1 display the phytochemical constituents of the methanol extract and its solvent fractions of *P. amarus*. All phytochemicals with the exception of balsams, resins and pholbotannins were present in the methanol extracts of *P. amarus.*

Table 2 quantifies the amounts of phytochemicals in the methanol extract and its solvent fractions. It shows that the alkaloid phytochemical in the methanol extract, is mostly isolated by the chloroform solvent.

Table 1: Qualitative analysis of phytochemicals in *Phyllanthus amarus* methanol extract and its fractions

Phytochemicals	ME	CF	EF	AF
Alkaloid	+++	+++	++	ND
Flavonoid	++	ND	+	+
Tannins	++	ND	+	+
Saponins	+	ND	+	+
Glycosides	++	ND	+	ND
Anthraquinones	+++	+	+	ND
Sterols	++	+	+	ND
Terpenes	++	++	++	+
Carbohydrates	+++	+	ND	+
Balsams	ND	ND	ND	ND
Resins	ND	ND	ND	ND
Phenols	+	+	+	+
Volatile oil	++	ND	ND	ND
Phlobatannin	ND	ND	ND	ND
Reducing sugar	+++	+++	++	++

+ Fairly present, ++ Highly present, +++ Very Highly present, ND: Not detected. + (0.01-0.99%), ++ (1.00-1.99%), +++ (≥ 2.00%), ND (0.00%), Awomukwu et al. (2014). ME= Methanol extract, CF= Chloroform fraction, EF= Ethanol fraction, AF= Aqueous fraction.

Table 2: Quantitative evaluation of phytochemicals in *Phyllanthus amarus* methanol extract and its solvent fractions

Phytochemicals	ME	CF	EF	AF
Alkaloid (%)	3.82 ± 0.53^{a}	2.96 ± 0.48^{a}	1.52 ± 0.24^{b}	ND
Flavonoid (%)	1.55 ± 0.26^{a}	ND	0.24 ± 0.08^{b}	0.49 ± 0.11^{b}
Tannins (%)	1.46 ± 0.12^{a}	ND	0.36 ± 0.07^{b}	0.28 ± 0.05^{b}
Saponins (%)	0.93 ± 0.03^{a}	ND	0.22 ± 0.04^{b}	0.61 ± 0.06^{a}
Anthraquinones (%)	2.51 ± 0.12^{a}	0.63 ± 0.07^{b}	1.38 ± 0.27^{c}	1.84 ± 0.17^{c}
Glycosides (%)	1.33 ± 0.14^{a}	ND	0.64 ± 0.03^{b}	0.86 ± 0.09^{b}
Phenols (%)	1.86 ± 0.012^{a}	1.06 ± 0.06^{a}	0.67 ± 0.07^{b}	0.43 ± 0.09^{b}

Data are expressed as Mean ± SD for triplicate determinations. ND: Not detected, ME= Methanol extract, CF= Chloroform fraction, EF= Ethanol fraction, AF= Aqueous fraction. Values bearing another superscript in a row differ significantly ($p\leq0.05$).

Toxicity report

The methanol extract and its solvent fractions showed no physical or behavioral signs of toxicity at all concentrations, even at the limit dose (5,000 mg/kg).

Antiplasmodial activity of extracts in early infection

Results in Table 3 explain that there exists a dose dependent reduction in parasitaemia and parasite suppression in mice treated with methanol extract and its solvent fractions. Highest parasite suppression was displayed by the highest dose of the chloroform fraction (100 mg/kg) of the methanol extract which compared well with the standard drug (Lonart®DS).

Table 3: Antiplasmodial activity of *Phyllanthus amarus* methanol extract and its solvent fractions in early infection.

Extract/Fraction	Dose (mg/kg)	Parasitaemia (%)	Parasite suppression (%)
Methanol extract	100	28	36
	300	26	41

	500	21	52
Chloroform fraction (CF)	50	22	50
	75	11	74
	100	6	87
Ethanol fraction (EF)	50	35	20
	75	19	58
	100	16	63
Aqueous fraction (AF)	50	36	18
	75	28	36
	100	23	48
Lonart®DS	20	4	92
Distilled water (mL/kg)	10	44	-

Lonart®DS= Artemether/Lumefantrine

Antiplasmodial activity of extracts in established infection

Daily progression of parasitaemia in experimental mice is illustrated in Figure 1. This figure shows that at Day 9 (*i.e.* 3 days after the 4-day treatment with *P. amarus* methanol leaf extract, chloroform fraction or Lonart®DS), parasitaemia in experimental mice had declined in a dose dependent manner, comparing Day 3 to Day 9 in the treated groups.

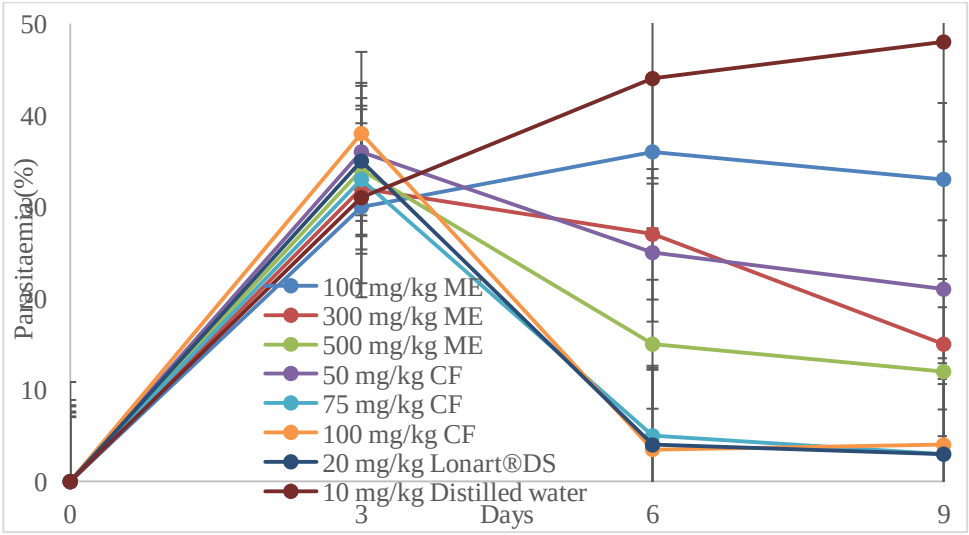

Figure 1: Antiplasmodial activity of *Phyllanthus amarus* methanol extract and its chloroform fraction in established infection.

Lonart®DS= Artemether/Lumefantrine

Plots in Figure 1 showed that there was no significant (p<0.05) difference between the antiplasmodial activity of the highest dose of chloroform fraction and the standard drug.

Prophylactic antiplasmodial activity

Antiplasmodial preventive activity of the methanol extract and the chloroform fraction is indicated in Table 4. Analysis showed that the chloroform fraction did not show great prophylactic activity even at the highest dose. However, methanol extract showed great activity, even greater than standard drug, pyrimethamine.

Table 4: Prophylactic antiplasmodial activity of *Phyllanthus amarus* methanol extract and its chloroform fraction.

Extract / Fraction	Dose (mg/kg)	Parasitaemia (%)	Parasite Suppression (%)
Methanol extract (ME)	100	8	71
	300	5	84
	500	2	93
Chloroform fraction (CF)	50	21	26
	75	11	61
	100	10	66
Pyrimethamine	1.2	4	87
Distilled water (mL/kg)	10	28	-

Pyrimethamine = Standard drug

Parasite suppression by the chloroform fraction in tissues

Table 5 indicaates parasite supression by the chloroform fraction in individaul tissues. While, Figure 2 shows parasite supression assessed at Day 0, 6 anf 9. Then, plots of mean survival time are displayed in Figure 3.

Table 5: Parasite suppression in tissues by *Phyllanthus amarus* chloroform fraction of the methanol extract in established malarial infection

Tissues	Water	Lonart® DS	Chloroform fraction (CF)			
			Doses (mg/kg)			
	10 mL/kg	20	50	75	100	0
		Suppression (%)				
Kidney		56	30	54	62	
Brain		56	62	67	73	
Liver		67	44	51	56	
Heart		55	64	69	76	
Pancreas		54	67	72	79	
Testis		7	14	32	41	
Epididymis		67	40	51	57	
Ovary		33	40	53	60	
Uterus		36	43	57	64	
Body weight reduction (%)	13 ± 2.4^{a}	7 ± 1.3^{b}	9 ± 1.2^{b}	6 ± 1.7^{b}	5 ± 1.9^{b}	$3 \pm 0.6^{c*}$
Mean survival time, MST (Day)	16 ± 2.02^{a}	28 ± 1.35^{b}	24 ± 2.55^{b}	27 ± 1.44^{b}	29 ± 0.92^{b}	30 ± 0.00^{b}

*Values with another superscript in a row differ significantly ($p\leq0.05$) *Increase*

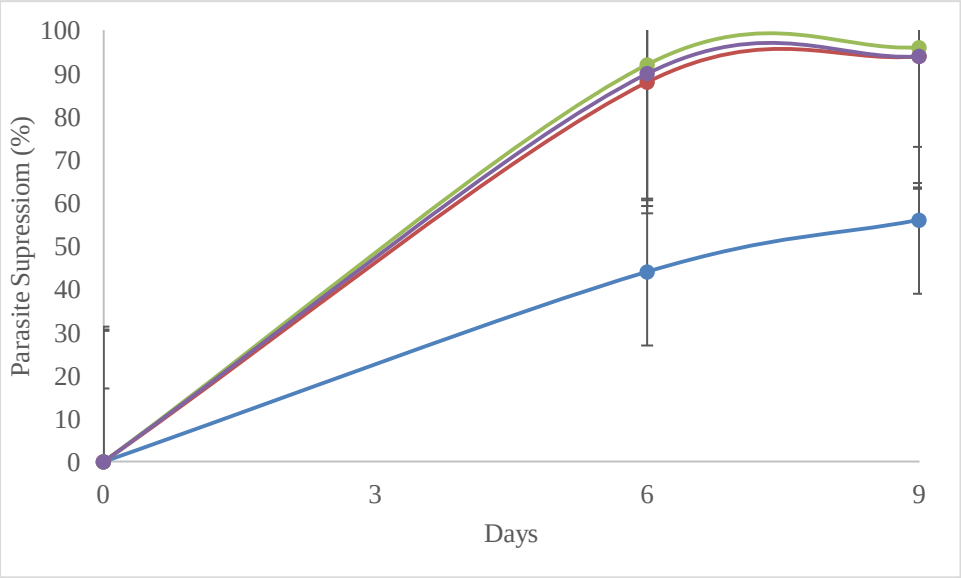

Figure 2: Parasite supression by chloroform fraction in blood.
Lonart®DS= Artemether/Lumefantrine

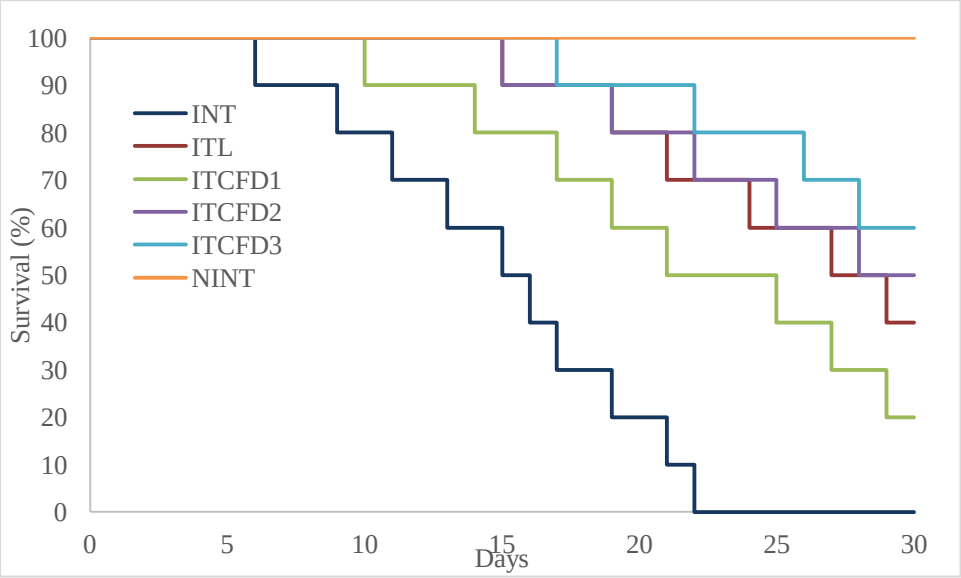

Figure 3: Effect of chloroform fraction on mean survival time in *P. berghei* infected mice.
NINT= Not infected, treated with water (10 mL/kg), INT= Infected, treated with water (10 mL/kg), ITL=Infected, treated with Lonart®DS (20 mg/kg), ITCFD1= Infected, treated with chloroform fraction dose 1 (50 mg/kg), ITCFD2= Infected, treated with chloroform fraction dose 2 (75 mg/kg), ITCFD3= Infected, treated with chloroform fraction dose 3 (100 mg/kg).

Haematological indices induced by chloroform fraction

Table 6 shows that there were significant decreases in PCV, WBC, RBC, Hb and neutrophils in infected mice when compared with uninfected mice. Graded doses of the chloroform fraction reverted the changes in these haematological indices in a dose dependent manner which compared well with the standard drug (Lonart®DS) treatment and positive control (uninfected mice). It is also important to note that, mice treated with highest dose of the chloroform fraction (100 mg/kg), showed a better improvement in comparison with mice treated with standard drug.

Table 6: Changes in hematological indices induced by *Phyllanthus amarus* chloroform faction of the methanol extract in established infection

Parameters (Hematology)	Treatment Doses (mg/kg)					
	Water		Lonart®DS	Chloroform Fraction		
	10 (mL/kg)		20	50	75	100
	Uninfected	Infected				
PCV (%)	36.5±2.1^{a}	22.5±2.5^{b}	33.5±3.5^{a}	27.8±3.2^{b}	29.4±3.2^{b}	35.2±2.7^{a}
WBC ($x10^{12}$/L)	7.4±2.2^{a}	5.8±1.2^{b}	6.4±0.1^{b}	6.1±0.3^{b}	6.2±0.7^{b}	6.8±1.3^{a}
RBC ($x10^{12}$/L)	6.1±0.3^{a}	3.8±0.4^{b}	5.6±0.6^{a}	4.4±1.1^{b}	5.4±0.6^{a}	6.2±0.9^{a}
Hb (g/dL)	12.2±0.5^{a}	7.5±0.8^{b}	11.2±1.2^{a}	9.8±1.5^{c}	10.7±1.6^{c}	11.8±1.4^{a}
Neutrophils (%)	6.5±1.5^{a}	4.5±2.5^{b}	5.0±2.0^{b}	4.8±2.3^{b}	5.1±1.3^{b}	5.5±1.7^{b}
Monocytes (%)	7.5±1.5^{a}	8.0±1.0^{a}	7.0±0.5^{a}	7.8±0.7^{a}	7.7±0.8^{a}	7.6±1.2^{a}
MCHC (g/dL)	33.4±5.2^{a}	33.3±4.8^{a}	33.4±3.2^{a}	35.3±5.6^{a}	36.4±6.2^{a}	33.5±4.1^{a}
MCV (FL)	59.8±6.3^{a}	59.2±5.6^{a}	59.8±4.9^{a}	63.2±7.2^{b}	54.4±6.1^{a}	56.8±3.8^{a}
MCH (pg.)	20.0±2.5^{a}	19.7±2.8^{a}	20.0±3.1^{a}	22.3±1.2^{a}	19.8±3.3^{a}	19.0±2.7^{a}

Results are expressed as Mean ± SD for n=5 mice/group. PCV= Packed Cell Volume, WBC= White Blood Cell, RBC= Red Blood Cell, Hb= Hemoglobin, MCH= Mean Corpuscular Hemoglobin, MCV= Mean Corpuscular Volume (MCV), MCHC= Mean Corpuscular Hemoglobin Concentration.. Values with another superscript in a row differ significantly ($p \leq 0.05$)

DISCUSSION

This study investigated the phytochemical constituents and associated antimalarial activity of *P. amarus* methanol extract and its solvent (chloroform, ethanol, aqueous) fractions in experimental mice. Phytochemical constituents are secondary metabolites of plants that serve as defense molecules against predation by lots of microorganisms, insects and other herbivores. These secondary metabolites are also responsible for the many medicinal and biological activities associated with herbal plants (Arun *et al*., 2012).

Phytochemical analysis of the methanol extract showed the presence of alkaloids, flavonoids, tannins, saponins, glycosides, anthraquinones, trepenes, sterols, carbohydrates, phenols, volatile oils and reducing sugars. Other studies have also confirmed the presence of alkaloids (Oshomoh and Uzama-Avenbuan, 2020; Saranraj and Sivasakthivelan, 2012), flavonoids (Oshomoh and Uzama-Avenbuan, 2020; Saranraj and Sivasakthivelan, 2012), phenols (Oshomoh and Uzama-Avenbuan, 2020; Saranraj and Sivasakthivelan, 2012), saponin (Oshomoh and Uzama-Avenbuan, 2020), terpenes (Simon *et al*., 2014), glycosides (Adegoke *et al*., 2010), anthraquinones (Bankole *et al*, 2019), carbohydrates (Dhongade and Chandewar, 2013), sterols (Dhongade and Chandewar, 2013) and reducing sugars (Egbon *et al*., 2017).

The methanol extract and its solvent fractions showed no signs of physical and behavioral changes or mortality in the course of the acute oral toxicity test when the animals were observed for two weeks. Lawson-Evi *et al*. (2008), Kushwaha *et* al. (2013), Adomi *et al*. (2017) and Ranjita *et al*. (2020) also reported zero toxicity of the extracts of *P. amarus* in experimental animals.

The antiplasmodial activity of the methanol and its solvent fractions assessed in early infection showed a dose dependent reduction in parasitaemia and increase in parasite suppression. Maximum suppression was expressed by the highest dose of the chloroform fraction (87 %). This suggests that chloroform is the best solvent for extraction, hence, selected for further study.

Phytochemical analysis of the chloroform fraction showed very high concentration of alkaloids. This agrees with the report of Bapna *et al*. (2014) who documented that the antiplasmodial activity of most extracts resides in alkaloids as they inhibit protein synthesis in the *Plasmodium* parasite.

So, the 4-day suppression assay of the methanol extract and the chloroform fraction was then assessed. Results from the study, again, indicated that maximum antiplasmodial activity resides in the chloroform fraction despite the methanol extract used at higher concentrations. The antiplasmodial activity of the extracts of *P. amarus* has been confirmed by other researchers. Ajala *et al*. (2015), reported the chemosupressive activities of the extracts of *P. amarus in vivo* (Aqueous extract= 81.27%; Ethanol extract= 78.23%). The *in vitro* study of Uzuegbu *et* al (2020) also demonstrated very high activity of the alkaloid (IC_{50}=0.27) and ethanol (IC_{50}=0.05) leaf extracts of the plant. Komlaga et al. (2015) reported the active antiplasmodial activity of the aqueous extract (IC_{50}=9.93 μg/ml and SI >10.1).

The 4-day parasite suppression assay indicates that the antiplasmodial activity of the chloroform fraction could be traceable to the alkaloids phytochemical, however, prophylactic antiplasmodial

activity was greater for the methanol extract (93 % parasite suppression at 500 mg/kg) in comparison with the chloroform fraction (66% parasite suppression at 100 mg/kg) and standard drug, pyrimethaime (86%). Ajala et al. (2011) reported an approximate 4 times chemo prophylactic decrease in parasitaemia in *Plasmodium yoelli* infected mice treated with 1600 mg/kg aqueous and ethanol extracts of *P. amarus.*

Assessment of parasite suppression of the chloroform fraction in individual tissues showed a dose dependent increase in every tissue. This is important as *Plasmodium* infection in tissues has been associated with a variety of damaging effects. Malaria infection has been linked severally to the generation of large amounts of reactive oxygen species (ROS) and thus, may lead to oxidative stress (Irighogbe et al. 2013). Accumulation of free radicals has been associated with loss or reduction of functions in various tissues of the body (Becker et al. 2004). Onyesom *et al.*, (2015) reported that extracts of *P. amarus* restored renal dysfunction induced by *P. berghei* infection. Onyesom *et al.* (2019) also documented that the extracts of *P. amarus* inhibited parasite multiplication in brain and liver cells of experimental mice. Ojezele et al. (2018) observed that treatment of malarial infected mice with *P. amarus* improved semen quality in infected mice.

Haematological changes associated with malarial infection include anaemia, thrombocytopenia, and disseminated intravascular coagulation (Maina et al. 2010; Chandra and Chandra, 2013). Our study shows that *P. berghei* infection in experimental mice reduced some hematological parameters including packed cell volume (PCV), white blood count (WBC), Red blood count (RBC), haemoglobin (Hb) and neutrophils and an increase in monocytes. These observations align with the report of other researchers. Reduced PCV (Adamu and Jigam 2019; Mutala et al. 2020; Al-Salahy et al, 2016), Hb (Adamu and Jigam, 2019; Mutala et al. 2020; Al-Salahy et al, 2016), neutrophils (Adamu and Jigam, 2019), RBC (Mutala et al. 2020), WBC (Al-Salahy et al, 2016) have been reported in humans infected with *Plasmodium falciparum*.

Depression of erythrocyte and haemoglobin levels in malarial infected cells is as a result of digestion of haemoglobin by malaria parasites (Becker et al., 2004). During the intraerythrocytic stage, the infection leads to the rupture of red blood cells (WHO, 1987), causing a reduction in the red cell number and thus, affects the haematocrit value. Effect of malarial infection on haemoglobin and red blood cells can cause reduction in the amount of oxygen delivered to body tissues and the quantity of carbon dioxide removed, since the number of haemoglobin and red blood cells are reduced. This can result in fatigue, causing muscle pain and convulsions (Wilson, 2008). Peripheral leukocyte or WBC counts have also been noted as being in the low to normal range during malaria, a phenomenon which is counterintuitive as one would expect increased production of WBCs during infection (McKenzie et al. 2005). Chandra and Chandra (2013), reported that low leukocyte count may be used as probable indicator for malaria in endemic countries.

However , the haematological indices in *P. berghei* infected mice as compared with test groups, show that administration of the chloroform fraction of *P. amarus* promoted a dose dependent increase in haematological parameters which were reduced by *Plasmodium* infection. This study can be related to the study of Nwakpa et al. (2014) who reported an increase of haematological parameters (RBCs, Hb, PCV) in mice infected with *Samonella typhi* and treated with extract of *P.*

amarus. Kolawole et al. (2019) also reported an increase in RBCs and WBCs in mice treated with the aqueous leaf extract.

CONCLUSION

The non-toxic chloroform fraction of the methanol extract of *P. amarus* had the highest alkaloid concentration and malarial curative activity, with moderate prophylactic potential. However, the methanol extract of the plant demonstrated maximum prophylactic activity. The prophylactic compounds in the methanol extract and the malarial curative alkaloid molecules in the chloroform fraction should be identified, and their synergistic effect studied. The investigation is pivotal to the discovery of antimalarial lead agents in *P. amarus,* which is commonly used in the Nigerian tradomedicine to treat malarial and associated infections.

Authors' Contributions

Theresa Ezedom and Joseph C. Mordi performed the laboratory work. Chinwendu Obogheneophruhe Elu analysed and interpreted data, and prepared the draft manuscript. Efe Aganbi and Joy O. Acha collected and processed the plant for analysis. They also analysed the constituents of plant's extract and administer same to the experimental animals. Innocent Onyesom conceived and designed the research, wrote the proposal, supervised all stages of the research and vetted the manuscript which was read by all authors and approved for submission.

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Conflicts of interest

None to declare.

Ethics statement

This study was approved by the Research and Bioethics Committee, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.

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References

Adamu, J., Jigam. A.A., 2019. Effects of malaria infection on some haematological and biochemical parameters in the general population and pregnant malaria patients attending two district hospitals in Niger State, Nigeria. Glob. J. Infect. Dis. Clin. Res. 5(1), 1-5.

Adegoke, A.A., Iberi, P.A., Akinpelu, D.A., Aiyegoro, O.A., Mboto, C., 2010. Studies on phytochemical screening and antimicrobial potentials of *Phyllanthus amarus* against multiple antibiotic resistant bacteria. Int. J. Appl. Res. Nat. Prod. 3(3), 6-12.

Adeneye, A.A., Benebo, A.S., Agbaje, E.O., 2006. Protective effect of the aqueous leaf and seed extract of *Phyllanthus amarus* on alcohol – induced hepatotoxity in rats. West Afr. J. Pharmacol. Drug Res. 22&23, 42-50.

Adomi, P.O., Owhe-Ureghe, U.B., Asagba, S.O., 2017. Evaluation of the toxicity of *Phyllanthus amarus* in wister albino rats. Afr. J. Cell. Path. 8, 27-35.

Ajala, T.O., Igwilo, C.I., Oreagba, I.A., Odeku, O.A., 2011. The antiplasmodial effect of the extracts and formulated capsules of *Phyllanthus amarus* on Plasmodium yoelii infection in mice. Asian Pac. J. Trop. Med. 2011, 283-287.

Ajiboye, B.O., Ibukun, E.O., Edobor, G., Ojo, A.O., Onikanni, S.A., 2013. Qualitative and quantitative analysis of phytochemicals in *Senecio biafrae* leaf. Int. J. Pharm. Investig. 1, 428-432.

Al-Salahy, M., Shnawa, B., Abed, G., Mandour, A., Al-Ezz, A., 2016. Parasitaemia and its relation to hematological parameters and liver function among patients malaria in Abs, Hajjah, Northwest Yemen. Interdiscip. Perspect. Infect. Dis. 2016, 1-5.

Arama, C., Troye-Blomberg, M., 2014. The path of malaria vaccine development, challenges and perspectives. J. Intern. Med. 275, 456–466.

Arun, T.B., Senthilkumar, K.P., Aarthy, A., 2012. GC-MS Determination of bioactive components of *Phyllanthus amarus* (L.) and its antibacterial activity. Journal of Pharmacy Research. 5, 9.

Awomukwu, D.A., Nyananyo, B.L., Onukwube, N., Uka, C.J., Okeke, C.U., Ikpeama, A.I., 2014. Comparative phytochemical constituents and pharmacognistic importance of the vegetative organs of some *Phyllanthus* species in South Eastern Nigeria. Int. J. Mod. Bot. 4(2), 29-39

Bankole, S.O., Akala, A.O., Adegboyega, D.A., Adekunle, E.A., 2019. Phytochemical screening of aqueous and methanolic extracts of *Phyllanthus amarus* and *Azadirachtha indica* leave. Int. J. Agric. Environ. Bio-res. 4(5), 305-315.

Banu, K.S., Cathrine, L., 2015. General techniques involved in phytochemical analysis. Int. J. Adv. Res. 2(4), 25-32.

Bapna, S., Choudhary, P.K., Satvekar, T., Ramaiya, M., Chowdhary, A., 2014. Antiplasmodial potential of crude alkaloid extract of three plants used in traditional medicine in India. Int. J. Pharm. Biol. 9(5), 48-51.

Becker, K., Tilley, L., Vennerstrom, J.L., Roberts, D., Rogerson, S., Ginsburg, H., 2004. Oxidative stress in malaria parasite-infected erythrocytes: Host-parasite interactions. Int. J. Parasitol. 34, 163–189.

Chandra, S., Chandra, H., 2013. Role of haematological parameters as an indicator of acute malarial infection in Uttarakhand state of India. Mediterr. J Hematol. Infect. Dis. 5(1), 1-7.

Dhongade, H., Chandewar, A., 2013. Pharmacognostical and phytochemical studies of *Phyllanthus amarus* leaves. Int. J. Biomed. Adv. Res. 4(6), 382-389.

Dondorp, A.M., Yeung, S., White, L., Nguon, C., Day, N.P., Socheat, D., Von, S.L., 2010 Artemisinin resistance: Current status and scenarios for containment. Nat. Rev. Microbiol. 8(4), 272-280.

Egbon, E.E., Olayioye, E.Y., Olayioye, S.A., Adesuyi, A.T., Owolabi, T., 2017.

Phytochemical screening and proximate composition of *Phyllanthus amarus*. Int. Res. J. Plant Sci. 8(1), 9-12.

El-Olemy, M.M., Al-Muhtadi, F.J., Afifi, A.F.A., 1994. Experimental phytochemistry: A laboratory manual. King Saud University Press, Saudi Arabia. Pp. 21-27.

Goel, N., Sirohi, S.K., Dwivedi, J., 2012. Estimation of total saponins and evaluate their effect on *in vitro* methanogenesis and rumen fermentation pattern in wheat straw based diet. J. Adv. Vet. Res. 2, 120-126.

Ifeoma, O., Samuel, O., Itohan, A.M., Adeola, S.O., 2013. Isolation, fractionation and evaluation of the antiplasmodial properties of *Phyllanthus niruri* resident in its chloroform fraction. Asian Pac. J. Trop. Med. 2013, 169-175.

Irighogbe, O.I., Agbaje, L.A., Aina, O.O., Ota, A.D., 2013. Oxidative stress and micronutrient therapy in malaria: an in vivo study in Plasmodium berghei in infected mice. Pak. J. Biol. Sci. 16(4), 160-167.

Kalra, B.S., Chawla, S., Gupta, P., Valecha, N., 2006. Screening of antimalarial drugs: An overview. Indian J. Pharmacol. 38(1), 5-12.

Khanna, A.K., Rizvi, F., Chander, R., 2002. Lipid lowering activity of *Phyllanthus niruri* in hyperglycaemic rats. J. Ethnopharmacol. 82(1), 19-22.

Kolawole, A.I., Blessing, N., Oyebimpe, A.F., Francis, O.S., 2019. Effects of aqueous leaf extracts of *Phyllanthus amarus* on liver function and blood parameters in male wister rats. J. Bio. Innov. 8(4), 471-483.

Komlaga, G., Cojean, S., Beniddir, M., Dicksona, R., Champy, P., Mensah, M.L, SuyyaghAlbouz, S., Jato, J., Loiseau, P.M., 2015. The antimalarial potential of three Ghanaian medicinal plants. Herb. Med. 1, 1-6.

Kumaran, A., Karunakaran, R., 2016. Anti oxidant and free radical scavenging activity of an aqueous extracts of *Coleus aromaticus*. Food Chem. 97, 109-114.

Kushwaha, S.K., Dashora, A., Dashora, N., Patel, J.R., Kori, M.L., 2013. Acute oral toxicity studies of the standardized methanolic extract of *Phyllanthus amarus* Schum & Thonn. J. Pharm. Res. 6(7), 720-724.

Lawson-Evi, P., Eklu-Gadegbeku, K., Agbonon, A., Aklikokou, K., Moukha, S., Creppy, E.E., Gbéassor, M. 2008. Toxicological assessment on extracts of *Phyllanthus amarus* Schum and Thonn. Sci. Res. Essay. 9, 410-415.

Maina, R.N., Walsh, D., Gaddy, C., Hongo, G., Waitumbi, J., Otieno, L., Jones, D., Ogutu B.R., 2010. Impact of *Plasmodium falciparum* infection on haematological parameters in children living in Western Kenya. Malar. J. 9(3), 1-11.

McKenzie, F.E., Prudhomme, W.A., Magill, A.J., Forney, J.R., Permpanich, B., Lucas, C.,Gasser, R.A., Wongsrichanalai, C., 2005. White blood cell counts and malaria. J. Infect. Dis. 192(2), 323–330.

Mutala, A.H., Badu, K., Owusu, C., Agordzo, S., Tweneboah, A., Abbas, D.A., Addo, M.G., 2020. Impact of malaria on haematological parameters of urban, peri-urban and rural residents in the Ashanti region of Ghana: a cross-sectional study. AAS Open Res. 2, 27-94

Obadoni, B.O., Ochuko, P.O. 2001. Phytochemical studies and comparative efficacy of the crude extracts of some homeostatic plants in Edo and Delta States of Nigeria. Glob. J. Pure. Appl. Sci. 8(2), 203–208.

Ojezele, M.O., Igbe, I., Okhuarobo, A., 2018. Reproductive indices in malaria infested mice treated with antimalarials, *Phyllanthus amarus* combined with vitamins. Bull. Fac. Pharm. Cario Univ. 56, 179–118.

Onyesom, I., Onumaechi, I.F., Ehiwario, J., Dagana, R., 2015. Antiplasmodial activity of *Phyllanthus amarus* preserves renal function in *Plasmodium berghei* infected mice. European J. Med. Plants. 5(1), 109-116.

Onyesom, I., Uzuegbu, U.E., Mordi, J.C., Moses, F.P., 2019. Alkaloid extract of *Phyllanthus amarus* (Stone Breaker) and associated antiplasmodial activity in experimental mice. Nigerian Journal of Biochemistry and Molecular Biology. 34(1&2), 1-7.

Oshiegbu, W., Elu, C.O., Onyesom, I., 2022. Profiling of seminal antioxidant indices and sperm quality in Plasmodium berghei-induced malarial mice treated with *Phyllanthus amarus*. Asian Pac. J. Reprod. 11(2), 84-92.

Oshomoh, E.O., Uzama-Avenbuan, O., 2020. Quantitative phytochemical composition and bioactive constituents of ethanolic extract of *Phyllanthus amarus* (schum. et thonn) leaves. Eur. J. Sci. Res. 2(4), 1-4.

Santhi, N.K., Sengottuvel, R., 2016. Qualitative and quantitative phytochemical analysis of *Moringa concanensis.* Int. J. Curr. Microbiol. App. Sci. 5(1), 633-640.

Saranraj, P., Sivasakthivelan, P., 2012. Screening of antibacterial activity of the medicinal plant, *Phyllanthus amarus* against urinary tract infection causing bacterial pathogens. Appl. J. Hyg. 1(3), 19 – 24.

Shingadia, D. 2014. Treating malaria in the UK. Paediatr. Child. Health. 24, 232–235.

Siddhuraju, P., Manian, S., 2007. The antioxidant activity and free radical scavenging capacity of dietary phenolic extracts from horse gram (*Macrotyloma uniflorum* (Lam.) Verdc.) seeds. Food Chem. 105(3), 950–958.

Simon, O.K., Simon, O.C., Okoh-Esene, R., Olutayo, O.O. 2014-2015. Biological and chemical evaluation of the extracts of the leaf of *Phyllanthus amarus Schum*. Int. J. ChemTech. Res. 7(5), 2347-2354.

Srividya, N. and Periwal, S. (1995). Diuretic, hypotensive and hypoglycaemic effect of *Phyllanthus amarus*. Indian Journal of Experimental Biology 33: 861-864.

Usunobun, U., Ngozi, O.P., 2016. Phytochemical analysis and proximate composition of *Vernonia amygdalina*. Int. J. Sci. World. 4(1), 11-14.

Uzuegbu, U.E., Opajobi, O.A., Utalor, J.E., Elu, C.O., Onyesom, I., 2020. Cytotoxicity and antiplasmodial activity of the alkaloid extract of 8 african plants. Thai J. Pharm. Sci. 44(4), 237-244.

Vuk, I., Rajic, Z., Zorc, B., 2008. Malaria and antimalarial drugs. Farm. Glas. 64, 51–60.

Wilson, D.D., 2008. McGraw-Hill's Manual of Laboratory and Diagnostic Tests. McGrawHill Companies, Inc. New York. 149.

World Health Organization (WHO) (1987) The Biology of Malaria Parasites. World Health Organization. Geneva. 33-44.

World Health Organization. 2022. *World Malaria Report* 2021. WHO, Geneva.