German J Pharm Biomaterials 2025; 4(3): 23-34
https://doi.org/10.5530/gjpb.2025.3.9

GJPB

Research Article

Exploring the leaf fractions of *Landolphia owariensis* P. Beauv. for bioactive constituents, *in vivo* and beta hematin inhibitory antimalarial activity

Chinelo Henrietta Okonkwo[1]*, Adaobi Chioma Ezike[1], Collins Azubuike Onyeto[1], Florence Nwakaego Mbaoji[1], Enitan Omobolanle Adesanya[2], Samuel Ayodele Egieyeh[3]

[1]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka, Enugu State, Nigeria.
[2]Department of Biochemistry, Olabisi Onabanjo University, Ago-Iwoye, Nigeria.
[3]Computational Pharmacology and Cheminformatics Research Group, Pharmacology and Clinical Pharmacy Unit, School of Pharmacy, University of the Western Cape, Cape Town, South Africa.

*Correspondence: chinokonkwo@gmail.com

Received: 01 May 2025; Revised: 25 July 2025; Accepted: 28 July 2025

Abstract

Malaria remains a global challenge even though it is preventable and curable. The study assessed the chemosuppressive efficacy and in vitro antiplasmodial activity of the methanol fraction and subfractions of *Landolphia owariensis* leaves. The subfractions of *Landolphia owariensis* leaves obtained from column separation of methanol fractions were investigated for chemosuppressive activity in mice and in vitro antiplasmodial activity using a beta-hematin inhibition assay. Cytotoxicity was determined using the Brine shrimp lethality assay (BSLA), while the active constituents were identified using GC-MS and NMR spectroscopy. The subfractions showed significant chemosuppressive effects against *P. berghei* at 100 mg/kg when compared with the negative control. The CF5 showed the highest parasite clearance and a chemosuppression of 88.14%. The beta hematin inhibitory activities of C9 (IC_{50} = 93.3 ± 0.05 µg/ml), $C9_A$ [octadecanoic acid (IC_{50} = 91.18 ± 0.07 µg/ml)] were comparable with chloroquine (IC_{50} = 89.7 ± 0.11 µg/ml). In the BSLA, C9 elicited medium cytotoxic activity (LD_{50} 190.6 ± 0.03 µg/ml). The GC-MS analysis of C9 identified 50 compounds, and the NMR spectrum of C9A indicated the presence of octadecanoic acid. The study suggests that the plant contains constituents that may be explored in the fight against malaria.

Keywords: *Landolphia owariensis*; *in vivo* antimalarial activity; beta hematin assay; BSLA

Introduction

Malaria is a parasitic infection that threatens the lives of people worldwide [1,2]. Current global estimates show that 608,000 people died of the disease in 2022. Millions of people in Sub-Saharan Africa are at risk of the disease, with Nigeria ranking highest (31%) in global malaria deaths. Children under five years are mainly affected by the disease [3]. Malaria is caused by the transmission of malaria parasites from female anopheles mosquitoes into human skin after a bite [4]. The parasites undergo several stages inside the host. At the intra-erythrocytic stage, the ingested haemoglobin is degraded in the parasite's food vacuole, releasing globin and free heme (Fe^{3+}), also known as ferriprotoporphyrin (FePPIX). For the parasite to remain alive in the host, free heme is rapidly detoxified into a biocrystal called hemozoin (beta-hematin), an important chemotherapeutic target in antimalarial drug discovery [5,6].

Historically, medicinal plants were the preferred primary options for treating diseases. Even with advances in medicine, medicinal plants remain a source of lead compounds for antimalarial drug development [7,8]. The antimalarial drugs that revolutionised the treatment of malaria were isolated from plants such as cinchona (quinine, chloroquine) and Artemisia annua (qinghao; artesunate) [9,10]. Although plants are beneficial and well tolerated, they contain different phytoconstituents that act at multiple biochemical targets [7]. A combination of two or more antimalarial drugs with different

mechanisms of action is the recommended treatment for malaria [11,12]. Despite the combination therapy, resistance to the existing antimalarial drugs limits the treatment goals, leading to increased morbidity and mortality associated with the disease [13,14]. This necessitates the tireless search for novel antimalarial molecules.

Landolphia owariensis P. Beauv is an ethnomedicinal plant famous for its folkloric use in treating several diseases. The plant is native to Africa and belongs to the family Apocynaceae [15]. Other researchers established the plant's potential as an analgesic, anti-inflammatory, antipyretic, and antimicrobial agent, which are important factors in controlling cellular damage associated with malaria [16,17]. A previous experiment with the plant revealed that the methanol fraction of the leaf extract exhibited the most significant *in vivo* antiplasmodial activity, including early infection, curative, and prophylactic activities [18]. Considering the plant's pharmacological potential, this study was designed to further assess the *in vivo* and *in vitro* antiplasmodial activity of the methanol subfractions of *L. owariensis*, to validate the antimalarial activity, and identify novel bioactive compounds.

Materials and Methods

Plant collection and identification

Fresh leaves of Landolphia owariensis were collected from Orba, Enugu State, Nigeria (6° 51' 24" N, 7° 23' 45" E). The plant material was definitively identified and authenticated at the International Centre for Ethnomedicine and Drug Development (InterCEDD) in Nsukka, Nigeria, by Mr. Anthony Ozioko. A herbarium specimen (InterCEDD/067) was duly deposited to support the research. The leaves were thoroughly cleaned, air-dried in the shade at room temperature, and then crushed into a coarse powder using a mortar and pestle, ensuring the retention of their essential properties for subsequent analysis.

Extraction and fractionation

The powdered plant material (1 kg) was macerated in absolute methanol for 48 h at room temperature (28 ± 1 °C) with occasional shaking. The resulting mixture was filtered through filter paper (No. 1), and the marc was washed repeatedly with fresh solvent until a clear filtrate was obtained. The filtrate thereafter evaporated under reduced pressure at 40 °C to dryness (Rotavapor, BUCHI 071, Switzerland). The crude methanol extract (60 gm) was subjected to solvent-guided fractionation using a separating funnel, starting with n-hexane, then ethyl acetate, and finally methanol, to yield the corresponding fractions. Subsequently, 5 gm of the methanol fraction (MF) was separated using open column chromatography (CC) with n-hexane > ethyl acetate> methanol, and, based on the similarity of the thin-layer chromatographic profiles, was pooled into five subfractions (CF1 - CF5).

We executed a comprehensive analysis of a 20 gm batch of the MF, which we successfully separated using column chromatography (CC) with a strategic sequence of n-hexane, ethyl acetate, dichloromethane, and methanol. This meticulous process yielded 15 distinct subfractions, labeled C1 to C15. We focused on subfractions C1, C4, C9, and C15, rigorously assessing their cytotoxicity using the brine shrimp lethality assay. The promising results from subfraction C9 led to further isolation into fractions $C9_A$-$C9_F$, which were also subjected to thorough antiplasmodial activity testing. Our findings indicated that $C9_A$ deserved in-depth characterization using Nuclear Magnetic Resonance (NMR). To ensure optimal preservation, all fractions were carefully stored in air-tight glass vials in the refrigerator, maintaining their integrity until they were ready for use.

Gas chromatography - mass spectroscopy analysis

The methanol fraction (C9) underwent rigorous analysis using a state-of-the-art gas chromatography-mass spectrometry (GC-MS) setup, featuring the advanced Agilent Technologies 7890 gas chromatograph paired with the Agilent 5975 mass spectrometer (Agilent Technologies, USA). This

analysis used a high-performance HP5-MS column with a length of 30 meters, an internal diameter of 0.320 mm, and a finely tuned film thickness of 0.25 μm. To ensure precise results, the oven temperature was expertly controlled, beginning at 80 °C and held for 2 min. It then ramped up rapidly to 120 °C per minute, ultimately reaching a maximum temperature of 240 °C, which was meticulously maintained for an additional 6 minutes. A 1 μl sample was injected, with the interface temperature between the gas chromatograph and the mass spectrometer set to 250 °C. This comprehensive analysis spanned mass ranges from 50 to 500, employing a splitless method to maximize sensitivity and accuracy.

Nuclear magnetic resonance spectroscopy analysis

The 1H and 13C NMR spectra were recorded at 400 MHz on a Bruker Ascend-500 spectrometer (Bruker Biospin Co., Karlsruhe, Germany). Deuterated chloroform with tetramethylsilane (TMS) as an internal standard was used to prepare the sample ($C9_A$) in 5 mm NMR tubes. The chemical shifts were expressed in parts per million (δ) according to the TMS signal. For better assignments, Non-Overhauser Effects Spectroscopy (NOESY) and Hetero Multi Quantum Coherence (HSQC) were applied.

Experimental animals

Adult Swiss albino mice of either gender (20 - 26 gm) were used for the study. The mice were obtained from and housed in steel cages at the Animal House of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, for 14 days prior to the study. They were maintained at a temperature of 28 ± 1 °C, 12 h light / dark cycle, relative humidity (14 ± 1%), with a regular rodent pelleted diet and liberal intake of water. An ethical clearance from the National Health Research Ethics Committee of the university (NHREC/05/01/2012A) was obtained. All procedures and techniques for the animal experiment complied with the National Institutes of Health guidelines for the use and care of laboratory animals (NIH, Department of Health and Human Services publication No. 85-23, revised 1985).

Parasites

A chloroquine-sensitive strain of *Plasmodium berghei* (NK 65) was obtained from the National Institute for Medical Research (NIMR), Lagos, Nigeria. The parasites were maintained by serial passage from infected mice to non-infected ones.

***In vivo* antimalarial activity study**

Parasite inoculation

An infected blood sample drawn from the tail vein of a donor mouse was diluted with normal saline (0.9% w/v) to obtain 1×10^7 *P. berghei* parasitised erythrocytes in 0.2 ml, as determined by measuring the percentage parasitaemia and the erythrocyte count. Each healthy experimental mouse was inoculated via the peritoneal route with 0.2 mL of infected blood.

Evaluation of activity on early malaria infection (4-Day Suppressive Test)

Evaluation of schizonticidal activity of the fractions against early *P. berghei* infection was performed as described by Peters and his coworkers [19]. The mice were weighed and randomly divided into 8 groups of 5 each. Treatment commenced orally 3 h post-inoculation, then continued for 4 consecutive days (D0 - D3) with a 24 h interval between doses. The treatment groups, CF1-CF5 and MF, received 100 mg/kg, respectively, while the control groups received either 3% Tween 80 (0.5 ml/kg) or artemether/lumefantrine (10 mg/kg). On the fifth day (D4), thin blood smears were taken from the tails of each mouse, fixed in methanol, and stained with 10% Giemsa solution at pH 7.2 for 10 min [20]. The

thin films were examined under a light microscope to identify parasitized erythrocytes per 100 cells in 4 random fields. The percentage parasitaemia level was estimated by comparing the parasitaemia in the control groups with the treated groups as follows:

% suppression = (pc- pt)/pc x 100

Where Pc = average parasitaemia in the control group

Pt = average parasitaemia in the treated group

Determination of body weight and temperature

The body weight of each mouse was recorded prior to infection (D_0) and on the fifth day (D_4) using a sensitive animal weighing balance (Griffin & George, London). Daily rectal temperature readings were taken in mice using a digital thermometer through D_4.

***In vitro* antiplasmodial studies**

Beta-hematin inhibition assay

The ability of the subfractions (C1 - C15) to inhibit the formation of beta-hematin *in vitro* was assessed using the method of Vargas and his coworkers [21] with slight modifications. A concentration of 4 mg/ml of the subfractions (C1 - C15) was tested in the beta hematin formation inhibition. Briefly, 10 µl of each of the subfractions and the standard drug (chloroquine) was dispensed in columns into 96-well plates. In addition, 10 µl of 1M HCl was dispensed into the 96-well plates containing the fractions. A freshly prepared 100 µl of hematin solution was added to the wells in rows A and B, followed by gentle shaking of the test plates for 10 min at 900 rpm. Also, 60 µl of saturated acetate solution already prewarmed (60 °C, pH 5.0) was added to all the wells. The test plate was incubated for 90 min at 60 °C. Thereafter, the wells in rows A and C received 750 µl of 15% pyridine, while the wells in rows B and D received 750 µl of HEPES at pH 7.5. The test plate was left to settle for 15 min after a 10 min gentle shake at 900 rpm. Finally, an aliquot of 100 µl was transferred to a sterilised 96-well plate in triplicate, and the absorbance was measured at 405 nm using a microplate reader.

Determination of the inhibition of beta hematin

Inhibition of beta (β) hematin was determined accordingly. The composition of the test samples is as follows:

$A_{Analysis}$; Blank (Control analysis) = hematin and 750 µl of HEPES (4.76 mg/ml)

$A_{Analysis}$ (Test analysis) = hematin and 15% pyridine

A_{CLT}; Blank (Blank control) = 15% pyridine only

A_{CLT} Blank; Blank (blank, blank) = 750 µl of HEPES only

The change in absorbance of the sample was determined using the formula below:

The residual absorbance ($\Delta A_{Analysis}$) as a result of inhibition of β-hematin;

$\Delta A_{Analysis} = A_{Analysis} - A_{Analysis}$; Blank

The residual absorbance (ΔA_{CLT}; Blank) free from the inhibition of the β-hematin;

ΔA_{CLT};Blank = A_{CLT};Blank – $A_{CLTBlank}$; Blank

The resulting inhibition of the β-hematin formation caused by the analysed sample;

$I_{Analysis} = \Delta A_{Analysis} - \Delta A_{CLT}$; Blank

An active sample is considered when $I_{Analysis}$ has a positive value, but a negative value indicates an inactive sample.

Cytotoxicity activity on brine shrimp lethality assay (BSLA)

Brine shrimp lethality bioassay was performed as a measure to assess cytotoxicity of the four most active subfractions (C1, C4, C9, and C15) of *Landolphia owariensis* based on the antiplasmodial activity results. The brine shrimp eggs were used in this assay, which was carried out according to the method reported by some researchers [22] with slight modifications. Each of the subfractions were dissolved in DMSO then prepared in different concentrations with artificial sea water from 1 to 1000 μg/ml in different test tubes.

Hatching of Artemia salina shrimps

Brine shrimp (Artemia salina) eggs were hatched in a vessel containing artificial seawater, which was prepared by dissolving 38 gm of sodium chloride in 1000 ml of distilled water. The solution was adjusted to pH 8.5 with 1N NaOH and aerated continuously for 48 h. Approximately 4.5 ml of the brine solution was added to each test tube. Appropriate dilutions of the subfractions (C1, C4, C9, and C15) were prepared according to their concentrations. Subsequently, each test tube containing the brine solution received 0.5 ml of the diluted test solution [23]. After hatching, ten shrimp nauplii were carefully transferred into each test tube using a glass capillary tube and were left in daylight at room temperature. The experiment was replicated three times, using only artificial seawater as a control. After 24 h, a magnifying lens was used to count both dead and surviving nauplii, and the results were recorded. Nauplii were considered dead if they did not exhibit any motion during observation. The mortality endpoint was defined as the inability of the nauplii to swim within 30 seconds of observation. The percentage lethality of the nauplii for each concentration, along with the control, was calculated by counting the number of live and dead nauplii in each test tube [24]. The 50% lethal dose (LD_{50}) values were then determined using GraphPad Prism (version 5.0).

Criteria for toxicity testing

The benchmark for validating toxicity testing of the fractions expressed as LD_{50} was by comparison to Clarkson's toxicity index which is classified as follows: LD_{50} greater than 1000 μg/ml is considered harmless, LD_{50} of 500 - 1000 μg/ml is weakly toxic, LD_{50} of 100 - 500 μg/ml is medium toxic, LD_{50} below 100 μg/ml is highly toxic [25].

Table 1. Effect of MF and sub-fractions of *L. owariensis* leaves on early infection against *P. berghei* infected mice.

Test substance	Dose (mg/kg) p.o	% Parasitaemia	% Suppression
CF1	100	10.10 ± 1.85*	80.20
CF2	100	26.90 ± 4.22*	47.25
CF3	100	7.35 ± 0.67	85.59
CF4	100	29.9 ± 5.99*	41.38
CF5	100	6.05 ± 1.14*	88.14
MF	100	14.7 ± 0.86	71.18
AL	10	4.13 ± 0.68*	91.90
Tween 80	-	51.00 ± 5.09	

n = 5; CF = sub-fraction; MF = methanol fractions; AL = artemether / lumefantrine *$P < 0.05$; values are presented as ± SEM

Results

Effect on 4-day suppressive test

The methanol fraction and its subfractions did not eliminate the parasites but reduced parasitaemia at a dose of 100 mg/kg body weight in the various groups of treated mice. The chemosuppression exhibited by CF5 (88.14%), CF3 (85.59%), and CF1 (80.20%) was higher than that of the MF (71.18%).

The CF5 had the highest chemosuppression, although lower than that of the standard drug (artemether/lumefantrine) at 10 mg/kg (91.90%), whereas CF4 induced the lowest parasitaemia suppression of 47.25% (Table 1). The reductions in parasitaemia observed in the treated groups (CFI, CF2, CF4, and CF5) were statistically significant ($P < 0.05$) compared with the negative control group.

Effect on body weight and temperature

The methanol fraction and the subfractions at 100 mg/kg body weight prevented the parasite-induced body weight reduction compared to the negative control after the 4-day suppressive test (Table 2). As shown in Figure 1, MF, CF2, CF3, CF4, and CF5 prevented a decrease in rectal temperature compared with the negative control, whereas CF1 caused a rise in rectal temperature.

Table 2. Effect of MF and subfractions of *L. owariensis* on body weight of *P. berghei* infected mice.

Test substance	Dose (mg/kg) p.o	Body Weight D_0 (g)	D_4 (g)	% Change
CF1	100	22.88 ± 0.70	24.96 ± 0.87	2.08
CF2	100	23.76 ± 0.59	24.38 ± 0.55	0.62
CF3	100	22.38 ± 0.66	24.38 ± 0.55	2.00
CF4	100	23.18 ± 0.56	24.00 ± 0.68	0.82
CF5	100	23.12 ± 0.78	23.82 ± 0.73	0.70
MF	100	21.82 ± 0.68	22.34 ± 1.06	0.52
AL	10	24.20 ± 0.84	25.34 ± 0.82	1.14
Vehicle		23.50 ± 0.92	21.32 ± 1.42	- 2.18

n = 5; CF = sub-fraction; MF = methanol fraction; AL = artemether / lumefantrine; values are presented as ± SEM

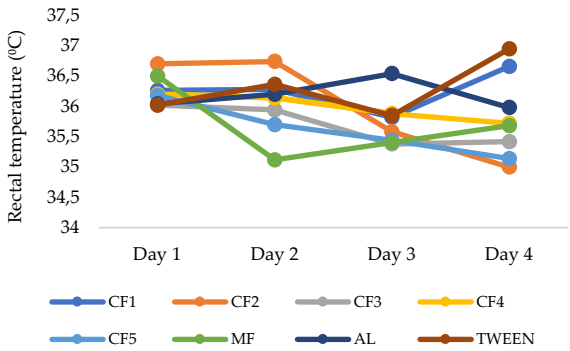

Figure 1. The effect of MF and subfractions of *L. owariensis* leaves on rectal temperature of P. berghei infected mice on 4-day suppressive test. n = 5; CF = subfraction; MF = methanol fraction; AL = artemether / lumefantrine; values are presented as ± SEM

Beta hematin Assay

The 50% inhibitory concentration (IC_{50}) value of the methanol subfractions (C1 - C15) ranged from 93.3 ± 0.05 µg/ml to 500 ± 0.11 µg/ml (Figure 2). C9 displayed the highest potency, with an IC_{50} value of 93.3 ± 0.05 µg/ml, which is comparable to chloroquine (89.7 ± 0.11 µg/ml), while C11 was the least active, with an IC_{50} value of 500 ± 0.05 µg/ml. From the quantitative IC_{50} testing, all subfractions showed appreciable inhibitory activity at 4mg/ml by inhibiting the formation of hemozoin, with IC_{50} values ranging from 91.18 ± 0.07 to 813.1 ± 0.09 µg/ml (Table 3). The highest inhibitory activity is shown by $C9_A$ (octadecanoic acid), with an IC_{50} value of 91.18 ± 0.07 µg/ml, which is comparable to chloroquine (IC_{50} = 89.7 ± 0.11 µg/ml).

Table 3. β-hematin IC_{50} (µg/mL) of the most active methanol subfraction compared to chloroquine.

Sub-fraction	IC_{50}
$C9_A$	91.18 ± 0.07
$C9_B$	111.3 ± 0.04
$C9_C$	225.0 ± 0.05
$C9_D$	133.9 ± 0.06
$C9_E$	282.3 ± 0.04
$C9_F$	813.1 ± 0.09
CQ	89.7 ± 0.11

Experiment was done in triplicates and values expressed as ± SEM; $C9_A$ - $C9_F$ = column fraction; CQ = chloroquine

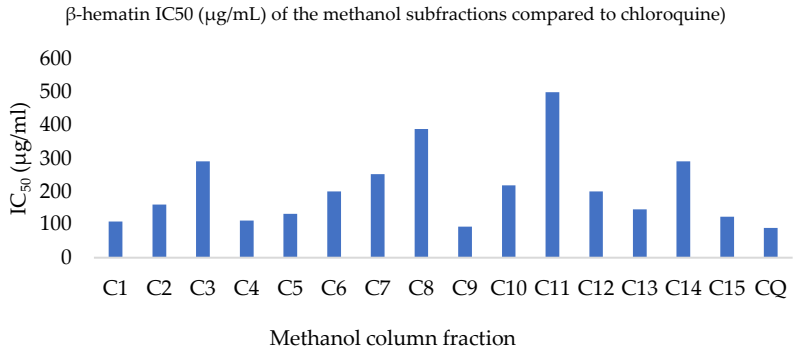

Figure 2. β-hematin IC_{50} (µg/ml) of the methanol subfractions compared to chloroquine. Experiment was done in triplicates and values expressed as ± SEM; C1 - C15 = methanol subfraction; CQ = chloroquine

Brine shrimp lethality assay

The four subfractions, C1, C4, C9, and C15, showed 100% mortality at a concentration of 1000 µg/ml, whereas cyclophosphamide showed 80% mortality. Mortality decreased with decreasing concentration, with the lowest mortality (0%) at 62.5 µg/ml; however, 5% mortality was observed in C9 at that concentration (Table 4). The results showed that all subfractions were moderately toxic, with LD_{50} values ranging from 168.9 ± 0.03 to 222.1 ± 0.01.

Table 4. Cytotoxicity result for brine shrimp lethality assay (BSLA) of the four most active subfractions.

Sample	% mortality at different concentrations (µg/ml)						LD_{50} (µg/ml)
	31.2	62.5	125	250	500	1000	
CI	0.00±0.00	0.00±0.00	40.00±0.00	65.00±0.00	100.0±0.00	100.0±0.00	168.9±0.03
C4	0.00±0.00	0.00±0.00	5.00±0.01	65.00±0.02	100.0±0.00	100.0±0.00	222.1±0.01
C9	0.00±0.00	5.00±0.00	15.00±0.00	65.00±0.01	100.0±0.00	100.0±0.00	190.6±0.03
C15	0.00±0.00	0.00±0.00	15.00±0.15	65.00±0.01	95.00±0.00	100.0±0.00	208.7±0.01
Cyclo	3.33±0.03	36.67±0.06	56.60±0.03	63.60±0.03	70.00±0.00	80.00±0.00	63.82±0.02

C1, C4, C9, C15 = subfraction, Cyclo = cyclophosphamide, LD_{50} = 50% effective lethal dose

Gas chromatography - mass spectroscopy analysis

Fifty peaks occurred from the GC-MS analysis indicating the constituents namely, hydroperoxide, 1-methylpentyl; cyclopentanol, 1-methyl-; decane; 1,3,5-cycloheptatriene 7,7-dimethyl-; 2,6-dimethyl-1,3,5,7-octatetraene, E,E-; o-cymene; 1-octanol, 2-methyl-; p-mentha-8-dehydeo-1,3-diene; 1,3,3a,6,7,9a-hexahydro-cis-cycloocta[c]furan; cyclododecane; 3-butenenitrile; 1-azabicyclo[3.2.0]hept-3-ene; decanedioic acid, didecyl ester; tetradecane; bicyclo[4.1.0]hept-3-ene-2,5-dione; 2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-5-phenyl-1-(trimethylsilyl)-; 1,4-cyclooctadiene; cyclopropane, nonyl-; 1,1,1-trifluoro-2-pentadecanol; undec-10-ynoic acid, undecyl ester; 12-crown-4; 2,4-di-tert-butylphenol; bicyclo[2.2.0]hexane-1-carboxaldehyde; 2-butenenitrile; cetene; (3E)-3-octen-1-yne, (E)-; tetracyclo[7.1.0(1,6).0(4,9).0(8,10)]decane; tetrazole, 5-(3,5-dimethylpyrazol-1-yl)-; carbonic acid, decyl nonyl ester; 2-norbornanemethanol, pentafluoropropionate; 1-octadecene; 2,5,10-undecatrienoic acid, methylester; 9-aza-bicyclo[4.2.1]nona-2,4-diene-9-carboxaldehyde; bicyclo[6.5.1]tetradec-1-ene, stereoisomer; 1,2,4-triazol-4-amine, 3-(3,5-dimethylpyrazol-1-yl)-; 7H-purin-6-amine, 7-methyl-; (9E)-9-icosene; hexaethylene glycol; butachlor; oxacyclohexadecan-2-one; heptaethylene glycol; cyclohex-2-enone, 3-(2H-tetrazol-5-ylamino)-; vinyl 10-undecenoate; bis(2-ethylhexyl) phthalate; cycloeicosane; cyclopropane carboxamide, 2-cyclopropyl-2-methyl-N-(1-cyclopropylethyl)-; liguloxide; pyrido[2,3-d]pyrimidine, 4-phenyl-; 9-aza-bicyclo[4.2.1]nona-2,4-diene and 2-phenyl-1,2-oxazolidine. The retention times of the compounds denoted by the various peaks are shown in (Figure 3).

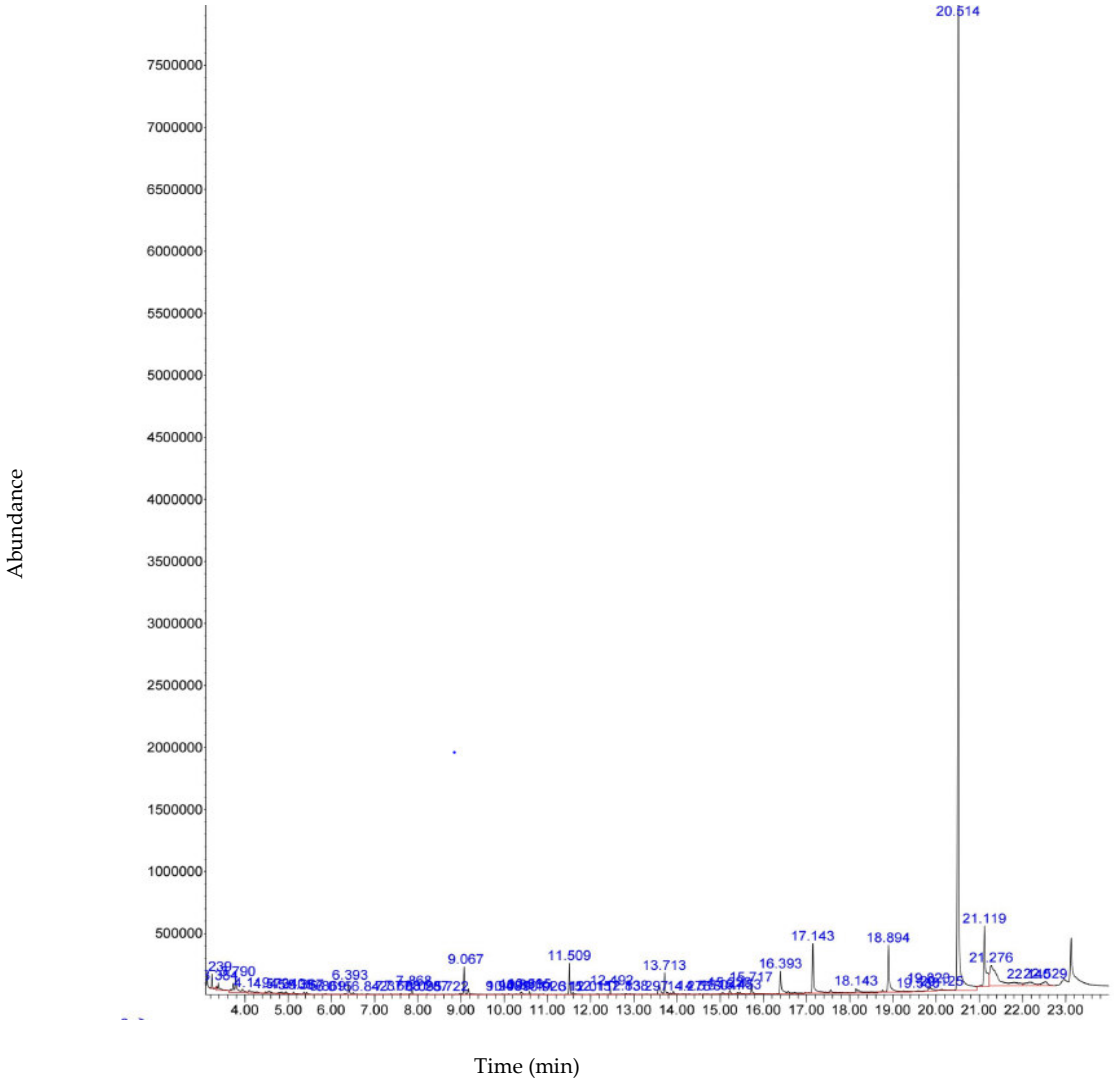

Figure 3. GC-MS chromatogram of C9 of *L. owariensis* showing 50 compounds identified.

NMR analysis

NMR analysis of C9A indicated the presence of long-chain fatty acid, octadecanoic acid (Figure 4), depicted by the crossing peak between 29.38 (^{13}C) and 1.18 (^{1}H) ppm (Supplementary Table S1). The physical properties include:

IUPAC name: Octadecanoic acid

Molecular weight: 290 g/mol-

Boiling point: 361°C

Melting point: 69.2 °C

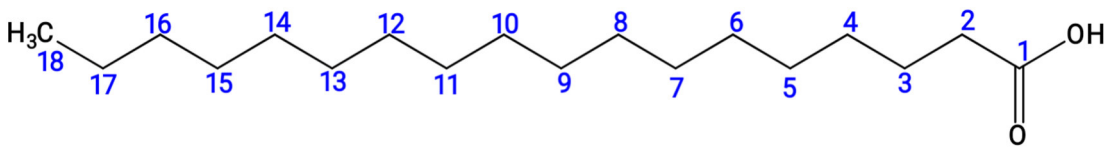

Figure 4. The chemical structure of Octadecanoic acid (Stearic acid).

Table S1. Nuclear magnetic resonance spectroscopy (NMR).

Carbon position	^{1}H-NMR (Experimental) δ (ppm)	^{13}C-NMR (Experimental) δ (ppm)	^{13}C-NMR (Reported) δ (ppm)**
1	-		178.74
2	2.28 (t, 2H)	32.76	33.81
3	1.18, bs	29.38-30.05	29.7–30.00
4	1.18, bs	29.38-30.05	29.7–30.00
5	1.18, bs	29.38-30.05	29.7–30.00
6	1.18, bs	29.38-30.05	29.7–30.00
7	1.18, bs	29.38-30.05	29.7–30.00
8	1.18, bs	29.38-30.05	29.7–30.00
9	1.18, bs	29.38-30.05	29.7–30.00
10	1.18, bs	29.38-30.05	29.7–30.00
11	1.18, bs	29.38-30.05	29.7–30.00
12	1.18, bs	29.38-30.05	29.7–30.00
13	1.18, bs	29.38-30.05	29.7–30.00
14	1.18, bs	29.38-30.05	29.7–30.00
15	1.18, bs	29.38-30.05	29.7–30.00
16	1.18, bs	31.94	31.91
17	1.65 (q, J = 8.0 Hz)	22.71	22.68
18	0.79 (t, 3H, J = 4.0 Hz)	14.14	14.11

**(Abdurrahman and Cai-Xiab, 2020)

Discussion

The burden of malaria is a serious menace to public health with a consequential socio-economic impact [26]. The 4-day suppressive test, which is typically used to determine the percentage inhibition of parasite growth, was employed in this study [27]. Test fractions that showed chemosuppressive activity above 30% are considered active against malaria, while chemosuppression at 100 mg/kg above 50% is considered very good [28,29]. Accordingly, the percentage parasitaemia obtained in this study revealed that the plant possesses antimalarial activity. This finding is consistent with a previous study reporting that the methanol extract and fractions of *L. owariensis* suppressed parasitaemia at higher doses [18].

Antimalarial compounds are expected to protect against weight loss caused by increased parasitaemia in infected mice. The methanol fraction and its subfractions not only prevented weight loss but also promoted body weight gain in the experimental mice. This implies that appetite-enhancing constituents may be present in the plant. Body weight loss and reduced appetite are notable symptoms of malaria infection in mice [30]. Rodents infected with parasitaemia suffer decreased metabolic rates and decreased internal body temperature, which could lead to death [31]. The methanol fraction and subfractions that did not protect the mice from a decrease in temperature indicate that the plant possesses constituents responsible for the hypothermic effect. The observed effect on temperature further substantiates the folkloric use of the plant in the treatment of malaria-induced pyrexia. However, CF1 demonstrated a temperature-stabilising effect and may be beneficial for boosting the immune system. The inconsistency in the results may be due to variations in the plant's phytochemical constituents and the pharmacodynamic makeup of the mice.

The β-hematin inhibition assay was explored to determine the in vitro antiplasmodial activity of the column fractions. The pronounced inhibition of β-hematin by the column fractions is highly appreciated, as this guided the isolation, purification, and antiplasmodial activity screening of the most active column fraction, which also showed considerable activity. This *in vitro* hemozoin-based colorimetric method is fast, inexpensive, replicable, and labour-saving compared to other assays for

screening antimalarial candidates [32]. Previous studies show that antimalarials such as chloroquine, amodiaquine, quinine, and mefloquine exhibit antimalarial activity through inhibition of β-hematin [33, 34]. The inhibition of β-hematin formation by the subfractions suggests that the plant's antimalarial activity is via the heme biomineralization pathway.

The brine shrimp lethality assay (BSLA) was used in the study as a first-line screen, supported by more advanced, specific cytotoxicity bioassays [35]. BSLA is a simple, fast, and less expensive technique that utilises small amounts (2-20 mg) of test materials and can detect biological activity in natural plant products. The medium toxicity displayed by all the subfractions may also be investigated for the management of cancer/tumour cells. This in vivo lethality test is a standard preliminary assay employed in the study of cytotoxic and antitumour agents [26]. It is noteworthy that the methanol stem extract of *L. owariensis* also showed a prominent cytotoxic effect on the human hepatocarcinoma HepG2 cell line, with an IC_{50} value of 40.5 ± 0.5 μg/ml [36]. Therefore, the plant has demonstrated potential for toxic properties.

The identification of active constituents is a prerequisite in the development of novel drugs from medicinal plants. The presence of 50 bioactive compounds identified by GC-MS analysis, with diverse pharmacological activities, buttresses the ethnomedicinal use of the plant in treating ailments. The bioactive compounds belonging to sesquiterpenes (liguloxide) and triunsaturated fatty acids (2,5,10-undecatrienoic acid, methylester) have significant pharmacological applications in the treatment of malaria [37,38]. The most abundant compound (bis(2-ethylhexyl) phthalate), indicated by the highest peak, exhibits mosquito larvicidal, antibacterial, antifungal, and cytotoxic activity [39]. In addition, compounds like 1,2,4-triazolo[4,3-a]pyridin-3(2H)-one, 5-methyl- and Pyrido[2,3-d] pyrimidine-4-phenyl possess anti-inflammatory, antipyretic, analgesic, and anticonvulsant properties that are crucial in controlling the cellular-damaging effects of malaria [40,41]. Besides the therapeutic potential of the compounds in the treatment or prevention of malaria, antitubercular and anticancer properties have been reported for 2,4-di-tert-butylphenol and 7H-purin-6-amine, 7-methyl, respectively [42,43]. However, the other compounds may be beneficial in the food, cosmetics, and chemical industries.

Although there are challenges in the identification of pure compounds from plant metabolites, the 13C NMR spectrum of the column fraction with the highest inhibition of beta hematin formation indicated the presence of octadecanoic acid (stearic acid) [44]. The pharmacological role of octadecanoic acid and its derivatives is gradually gaining attention as compounds involved in plants' antiplasmodial activities [45,46]. This strongly supports the present study, in which octadecanoic acid demonstrated antiplasmodial activity by inhibiting the formation of hemozoin.

Conclusion

Our findings indicate that the methanol fractions of *L. owariensis* possess constituents that may account for the antimalarial activity. The *in vivo* and *in vitro* antimalarial activity of the bioactive compounds was mediated by parasitaemia suppression and inhibition of haemozoin formation. The current evidence supports the ethnomedicinal use of the plant's leaves for malaria treatment. Some of the bioactive compounds identified provide opportunities for novel drug candidates. The scientific significance of the findings underscores the need to validate the bioactive compounds further using additional assays for antimalarial drug development. However, computational approaches are underway to predict drug-target interactions, druglikeness, and the safety profiles of potential antimalarial compounds.

Author's contributions

CHO: design, methodology, and writing. ACE: supervision, review, and editing. CAO: analysis, review, and editing. FNM: analysis, review, and editing; EOA: methodology, investigation, and review. SAE: methodology, investigation, and review. All authors gave final approval for the work to be published.

Competing interests

The authors declare that they have no competing interests.

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How to cite this article:
Okonkwo CH, Ezike AC, Onyeto CA, Mbaoji FN, Adesanya EO, Egieyeh SA. Exploring the leaf fractions of Landolphia owariensis P. Beauv. for bioactive constituents, in vivo and beta hematin inhibitory antimalarial activity. German J Pharm Biomaterials. 2025;4(3):23-34.