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Phytochemicals, Antioxidant and Antimalarial Activities of *Combretum micranthum* G. Don. Leaves

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ABSTRACT
In Nigeria, the identification of novel compounds for the development of more effective and safe treatments to fight malaria is urgently needed. Medicinal plants are one of the main opportunities in the discovery of such new compounds. *Combretum micranthum* (Combretaceae) is a medicinal plant widely known and used in Africa to treat a variety of conditions such as diabetes, fever, coughs, bronchitis, diarrhea, pain, malaria and liver disorders, among others. The study was aimed to evaluate the phytochemical, antioxidant, antimalarial and acute toxicity studies of *Combretum micranthum* leaves. Phytochemical and antimalarial evaluations were carried out using the standard methods. The antioxidant activities of the methanol extract was determined by means of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Alkaloid, flavonoids, saponins, tannins, glycosides, phenols, triterpenes and steroids while anthraquinones was absent in the *Combretum micranthum* leaf extract. The DPPH radical scavenging ability of the extract showed the following trend methanol extract < Ascorbic acid. The inhibitory effects of *Combretum micranthum* leaves crude extract against *P. falciparum* at concentrations of 500, 250, 125, 62.5 and 31.25 mg/ml with percentage growth inhibitions of 88.8-66.6 %. The FTIR analysis showed the presence of alcohol, phenol, carboxylic acid, aldehyde and ketone functional groups. GCMS analysis revealed different compounds with antioxidant and antimalarial properties. The LD_{50} of *Combretum micranthum* leaves was found to be greater than 5000 mg /kg and could be considered practically safe for consumption. The extract was found to possess constituents that may be associated with its antioxidant and antimalarial effects observed at doses tested.

Keywords*:* Acute toxicity; Antimalarial; Antioxidant; *Combretum micranthum*; Phytochemical

INTRODUCTION

In developing countries, the drugs used to treat most diseases are imported and too expensive. Recently, the drugs are out of reach of populations because the pharmaceutical firms have appropriated their production; so that about 90% of peoples in Africa are moving away from modern health facilities and turn to traditional medicine (Baba-Moussa *et al.,* 2016). Search for new antimalarial and antioxidants sources remain a challenge for pharmaceutical and research institutions. Traditional medicine is one of the major tracks that could be explored through its medicinal plants (Baba-Moussa *et al.,* 2016). Despite recent interest in molecular modelling and other synthetic chemistry techniques by pharmaceutical companies, natural products especially medicinal plants remain an important source of new medicines (Mabozou *et al.,* 2019). The use of herbal medicines to prevent the genesis and complications of malaria offers new alternative since synthetic molecules pose problems (Mabozou *et al.,* 2019).

It is therefore essential to identify natural products to counter the progression of malaria. Nowadays, much of attention has been given to the use of phytochemicals as a protective strategy against malaria and oxidative stress (Baba-Moussa *et al.,* 2016).
Phenolic compounds were efficient in inhibiting reactive oxygen species induced organ pathologies (Mabozou *et al.,* 2019). Furthermore, there is an increasing preference for natural antioxidant rather than synthetic compounds because of the safety of the natural sources. Plants are known to provide a source of inspiration for novel drug compounds and this is sequel to the fact that medicines derived from plants have made large contributions to human health and well-being (Mabozou *et al.,* 2019). Many herbal medicines are known to have various types of polyphenolic compounds and may be quite safe and effective in reducing malarial and oxidative stress effects (Mabozou *et al.,* 2019).
Plasmodium falciparum, among the protozoan species of the genus *Plasmodium*, causes most of the severe cases of this ailment (Akanji *et al.,* 2016). Many drugs have been used to treat malaria e.g. quinine, chloroquine, mefloquine, artemisinin amongst others, but the parasite has developed resistance against a lot of these drugs (Akanji *et al.,* 2016). In the search for new therapeutic substances, a great number of researchers have resorted to plant sources. This is due to the fact that many of these plants are used in African traditional medicine (ATM) and drugs from natural products have been summon for use as origin of development of new antimalarials so as to eradicate drug resistance problems (Akanji *et al.,* 2016).
Combretum micranthum is an important medicinal plant in West Africa known for the moderate antiplasmodial activity of its leaves (Keita *et al.,* 2020). The leaves are used to make the popular 'quanqueliba' drink', a refreshing tea traded as 'kinkéliba'. The seeds are edible and the leaves are used as fodder for small ruminants. Leaves, roots and barks have many medicinal uses (antipyretic, tonic, diuretic, antidiarrhoeal and choleretic) (MSBP, 2007). Leaf extracts have been found to exhibit anti-viral and anti-inflammatory properties. It is also commonly used in Africa by native healers for the treatment of *malaria* (MSBP, 2007). Natural products continue to play a major role in drug discovery and development, and medicinal plants have been a rich source of many compounds with large production of bioactive molecules (Keita *et al.,* 2020). Therefore, the next generation antimalarials or the scaffolds necessary for their synthesis may be found in plants currently used in African traditional medicine against malaria

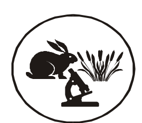
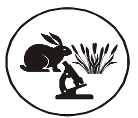
MATERIALS AND METHODS

Collection and Identification of Plant Materials

The leaves of *Combretum micranthum* were collected from local farm in March, 2021 at Dambatta Local Government Area, Kano State, Nigeria. The plant was identified and authenticated in the herbarium of the Plant Biology Department of Bayero University, Kano, Kano State, Nigeria and a voucher specimen number was deposited.

Preparation of Plant extracts

The leaves of the plant was cleaned, air dried and ground to coarse powder using grinding machine. The powder was stored in air tight containers for further use. One hundred grams (100 g) of the powdered leaves were soaked into 1000 ml of methanol. The mixture was allowed to stand for 3 days at room temperature (28 ±2ºC) with hourly agitations. The extract was sieved through a muslin cloth, filtered through a Whatman (No.1) filter paper, poured unto a clean evaporating dish and placed on a water bath at 50ºC until all the solvent evaporated.

Qualitative Phytochemical screening of Methanolic extract of Combretum micranthum Leaves

The plant extract was subjected to phytochemical screening in order to identify the phytochemical constituents of the plant using the methods described below.

Tests for carbohydrates

Molish's (General) Test for Carbohydrates

To 1 ml of the filtrate, 1 ml of Molish's reagent was added in a test tube, followed by 1 ml of concentrated sulphuric acid down the test tube to form a lower layer. A reddish colour at the interfacial ring indicates the presence of carbohydrate (Evans, 2009).

Tests for Saponins

Frothing test

About 10ml of distilled water was added to a portion of the extract and was shaken vigorously for 30 seconds. The tube was allowed to stand in a vertical position and was observed for 30 mins. A honeycomb froth that persists for 10-15 mins indicates presence of saponins (Evans, 2009).

Test for Flavonoids

Shinoda Test

A portion of the extract was dissolved in 1-2 ml of 50% methanol in the presence of heated metallic magnesium chips and a few drops of concentrated hydrochloric acid were added. Appearance of red color indicates the presence of flavonoids (Evan, 2009).

Test for Alkaloids

Wagner's Test

Few drops of Wagner's reagent was added into a portion of the extract, whitish precipitate indicates the presence of alkaloids (Evans, 2009).

Test for Steroids and Triterpenes

Liebermann-Burchard's test

Equal volumes of acetic acid anhydride was added to the portion of the extract and mixed gently. Concentrated sulphuric acid (1 ml) was added down the side of the test tube to form a lower layer. A colour change observed immediately and later indicates the presence of steroid and triterpenes. Red, pink or purple colour indicates the presence of triterpenes while blue or blue green indicates steroids (Evans, 2009).

Test for Cardiac Glycosides

Kella-killiani's test

A portion of the extract was dissolved in 1ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred into a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. Interphase for purple-brown ring was carefully observed, this indicates the presence of deoxy sugars and pale green colour in the upper acetic acid layer indicates the presence of cardiac glycosides (Evans, 2009).

Test for Tannins

Ferric chloride test

Exactly 3-5 drops of ferric chloride solution was added to the portion of the extract. A greenish black precipitate indicates the presence of condensed tannins while hydrolysable tannins give a blue or brownish blue precipitates (Evans, 2009).

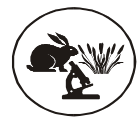
Test for Anthraquinones

Borntrager's test:

Exactly 5 ml of chloroform was added to the portion of the extract in a dry test tube and shaken for at least 5 mins. This was filtered, and the filtrate shaken with equal volume of 10% ammonium solution, bright pink colour in the aqueous upper layer indicates the presence of free anthraquinones (Evans, 2009).

Antioxidant activity Procedure

The antioxidant activity of the *Combretum micranthum* leaves extract was measured in terms of radical scavenging ability, using a stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the modified method adopted from (Sani and Dailami, 2015).



200µl of 100µM methanol solution of DPPH were added to 100µL of various concentrations of the sample fractions in methanol (1000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.8µg/ml) and made to react in dark for 30mins time at room temperature. Absorbance of the blank, test and control were recorded at 517 nm. The experiment was performed in triplicate and scavenging activity was calculated by using the following formula and expressed as percentage of inhibition.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

The concentration corresponding to the 50% inhibition (IC_{50}) was determined using probit analysis by means of SPSS 16.0 software. The IC_{50} values obtained are compared with that of ascorbic acid as a standard antioxidant.

Collection of the Test Organisms

Test organisms (*Plasmodium falciparum*) were obtained from the Laboratory of Bayero University Kano old campus clinic. The organisms were obtained from the infected blood samples of patients attending General Outpatient Department unit with malaria infection. This was confirmed microscopically by placing a drop of blood sample on a clean grease free slide to form a head and tail film. The film was fixed using absolute Ethanol, allowed to air dry and stained with 10% Giemsa solution for 30mins then observed using oil immersion objective lens in compound light microscope (Cheesbrough, 1987).

Preparation of the Culture Medium for Cultivation of Malarial Parasites

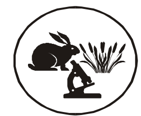
Cultivation of malarial parasites was performed using Candle Jar Method developed by Trager and Jensen (1976). RPMI 1640 medium (containing 25Mm of HEPES buffer, glucose) was used. One packet (about 10.4g) of RPMI 1640 was dissolved in 960ml of double distilled water and 40µg/ml of gentamycin sulfate was added. This solution was passed through a Millipore filter of 0.22µm porosity and sterilized at 121^0C for 15mins in an autoclave. This was then stored at 4^oC in refrigerator as 96ml aliquots in glass media bottle.

Washing Medium (incomplete medium), Serum Preparation and formation of the Complete Medium

The washing medium (incomplete medium) was achieved by adding 4.2 ml of 5% sodium bicarbonate to the 96 ml of stock RPMI 1640 medium. To obtained complete medium, blood group O Rhesus positive was collected from the blood bank of Sir Muhammad Sunusi Specialist Hospital in a plain container and kept at 4° C and then centrifuged at 10,000rpm for 20mins, at 4° C. The serum was separated aseptically and kept in aliquots, then inactivated by keeping it at 56° C water bath for half an hour. After inactivating the serum, 10ml of the inactivated serum was added to 90 ml of the incomplete medium to get a complete enriched medium for culture. The inactivated serum was stored in deep freezer at -20° C/-70° C before use (Fairlamb *et al.,* 1985).

Preparation of Infected Erythrocytes (RBCs) for Culture

Parasites used for the study were field specimens obtained from the patient/subjects positive to malaria. The parasites were confirmed to be *P. falciparum* based on the slide smear observation of the positive slide by the clinic laboratory scientist. The confirmed infected blood samples were collected and transferred into centrifuge tubes containing anticoagulant (EDTA). Then this were centrifuged at 1500 rpm for 10 min at room temperature. Plasma and fats were removed with the sterile Pasteur pipette. After this, washing medium was added, centrifuged at 1500 rpm for 10min and the supernatant was removed.



This washing process was repeated three times after which equal amount of complete medium was added to the sediments (parasitized red blood cells) and stored at 4^0 C (Osisanya *et al.,* 1981).

Continuous Culture of the Plasmodium falciparum

The parasites were then obtained for continuous culture after centrifugation of the original blood specimen (infected blood sample) obtained. The infected RBCs obtained were washed following the modified Trager and Jensen, (1976) protocol. For initiation of culture, suspension (50%) of infected cells with complete medium (with 15% inactivated serum) was prepared. Appropriate amount of uninfected cells were added to get an initial parasitaemia of 3 to 5% and diluted with complete medium to get 5% cell suspension. The culture was kept in a Candle Jar incubator at 37ºC.

The culture was monitored after every 24 hrs, the medium was removed using a sterile Pasteur pipette without disturbing the parasitized cells that settled down. The parasitized cells were then mixed without frothing, a drop of blood was placed on the slide and a thin film was made. Fresh complete media (with 10% serum) was added, mixed properly and kept back in the incubator (Fairlamb *et al.,* 1985) and the film was stained and examined microscopically following the methods described by Cheesbrough (1987).

Preparation of Different Concentrations of the Test Extract

Following the method of Ekwenye and Elegalam (2005) the concentration of 500 mg/ml of both extracts were prepared by dissolving 1 g of each extract in 1ml of dimethyl sulfoxide (DMSO) to form the stock solutions of 1000 mg/ml. From the stock solution of each extract, concentrations of 500, 250, 125, 62.5 and 31.25 mg/ml were prepared by double dilution procedure using serial dilution method (Aneja, 2005).

Preparation of the Orthodox Drug {Artemether injection (positive control

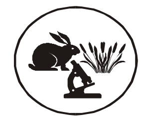
Stock solution 80 mg/ml of Artemeter (Clarion ARTE-JECT Brand) was prepared in 70% dimethyl sulfoxide (DMSO) according to the procedure of Noedl *et al.,* (2002). Subsequent dilutions (serial dilution) were made using incomplete RPMI 1640 medium as a diluent to yield a desired two fold concentration for the artemeter. Fifty microliter of the solution was transferred to 96 well micro culture plates. The plates were dried in an incubator at 37ºC and stored at 4ºC in sterile plastic container before use in accordance with the procedure of Russell *et al.,* (2003).

Anti-malarial Activity of the Crude Extract against P. falciparum Culture

The anti-malarial activity of *Combretum micranthum* leaves extract was evaluated *in vitro* against *Plasmodium falciparum* grown on RPMI 1640 medium at various concentrations of the extract. The amount of the medium, crude extracts and infected red blood cells sample (test organisms) were in ratio of 8:1:1 in the culture vials (WHO, 2015). The DMSO was used as negative control (drugs free well) accordingly, and artemether injection was used as positive control. All vials were kept in a Candle Jar incubator and were incubated at 37°C. The percentage parasitaemia were determined microscopically using thin blood smear after 72 hours intervals of the extracts and parasites contact. The percentage parasitaemia levels of both crude extracts in the tests vials culture at various concentrations were recorded and the percentage growth inhibition were calculated using percentage parasitaemia of negative control and that of the tests vials culture at various concentrations (Benoit, 1996).

Determination of Percentage Parasitaemia and Growth Inhibition (%GI)

The percentage of parasitaemia was determined using the methods adopted by Kalra *et al.* (2006). From the test vials, thin smears were prepared on slides.



The slides were allowed to dry and then fixed with absolute ethanol. After fixing, the slides were allowed to dry and then stained with 10% Giemsa in methanol for 30mins. After 30mins the slides were rinsed with water and then allowed to air dry. To estimate the percentage of red blood cells infected with *Plasmodium falciparum,* the slides were carefully observed under microscope using ×100 oil immersion objective lens in five different fields on each slide. The percentage parasitaemia were calculated using the following formula.

% Parasitaemia = Number of parasitized R B C ∕ Total number of R B C × 100

The percentage of growth inhibition (% GI) of each concentration of the crude extract was determined using the following equation:

% GI= a – b÷a × 100

Where "a" stands for mean % parasitaemia of negative control (drugs free well) vials and "b" stands for mean % parasitaemia of treated vials, for comparing GI in treated vials and negative control vials (WHO, 2015).

Fourier Transformed Infrared Spectroscopy Analysis (FTIR)

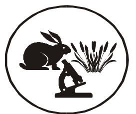
Air-dried sample of methanol extract from selected plant species was analyzed for identification of characteristic functional groups using Fourier Transform Infrared (FT-IR) spectrophotometer (Shimadzu 8400) at the Multi-User Laboratory, Department of Chemistry, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. A small quantity (0.1g) of extract sample and 0.025g of dry potassium bromide (KBr) were homogenized using mortar and pestle. A portion of the homogenized mixture was placed on the disc and pressed using a mini hand press to form a KBr thin film and the disc was placed in the FT-IR spectrophotometer in which spectra was measured by accumulating 64 scans at 4 cm-1 resolution in the spectral range of 4000 to 400 cm-1. Percentage transmittance was plotted against wavelengths. The FT-IR spectra were used to identify the functional groups of active metabolites based on the peak values in the infra red region.

Gas chromatography-mass spectrum (GC-MS) analysis

GC-MS analysis was conducted at the Multi-User Laboratory, Department of Chemistry, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. The extract was diluted to 1 mg/mL and 0.5 µL was separated on non-polar DB5-HT capillary column (20 m × 0.18 mm) with 0.1 µm film (J and W Scientific) fitted to an AutoSystem XL GC-MS. The injector temperature was 270°C and the oven temperature was programmed at an initial temperature of 50° C for 1 min, rising at 25°C per minute to 160°C and maintained at that temperature for 1 min. The temperature was subsequently increased by 10°C per minute to 300°C and maintained at that temperature for a further 4.6 minutes. The carrier gas was helium at a constant pressure of 5 kPa. The GC was directly interfaced with AutoSystem XL quadrapole mass spectrometer with an interface temperature of 270°C. Sample ionisation was by 70 eV electron impact and was analysed in positive mode. Structural determination was by comparison of mass spectral patterns to ChemSpider data bases (Ian and Frederick, 2012).

Acute toxicity studies of methanol extract of Combretum micranthum Leaves (Lethal Dose (LD_{50} Determination)

The method of Lorke (1983) was employed. Thus, the phase I involved the oral administration of three different doses of 10, 100 and 1,000 mg/kg of the crude extract, to three different groups of three adult Wister albino rats. In a fourth group, three adult male Wilter albino rats were administered with equivalent/volume of distilled water to serve as control. All the animals were orally administered with the extract using a curved needle to which a catheter had been fixed.



The animals were monitored closely every 30 minutes for the first 3 hours after administration of the crude extract, and then hourly for the next 6 hours for any adverse effects. Then the animals were left for 72 hours for further observation.
When no death occurred, the phase II was employed, where only one animal was required in each group. Groups 1–4, animals were orally given 1,500, 2,200, 3250 and 5,000mg/kg dose levels of the crude extract. All the animals were left for observation as in stage one.

RESULTS

Phytochemical screening of methanol extract revealed the presence of alkaloid, flavonoids, saponins, tannins, glycosides, phenols, triterpenes and steroids while anthraquinones were absent (Table 1).

Table 1: Qualitative Phytochemical screening of Methanolic extract of *Combretum micranthum* leaves

Metabolite	Inference
Alkaloid	+
Flavonoid	+
Saponins	+
Cardiac glycoside	+
Tannins	+
Steroids	+
Triterpenes	+
Phenols	+
Anthraquinones	**-**
Carbohydrate	+

Key: + Present ; - Absent

Free radical scavenging ability of the methanol extract of *Combretum micranthum* leaves was evaluated using DPPH radical. Ascorbic acid was used as positive control. It was determined that methanol extract of *Combretum micranthum* leaves possessed higher radical scavenging ability of 98.1 % at the highest concentration of 1000 µg/mL and was compared with standard where it showed 99.7% activity (Table 2).

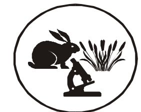
Table 2. Antioxidant activity of Methanol extract of *Combretum micranthum* leaves

Analyte	Concentration (µg/mL) / % Inhibition					
	1000	500	250	125	62.5	31.25
Methanol extract	98.1	88.9	83.2	79.8	79.1	77.9
Ascorbic acid	99.7	99.3	98.7	97.4	95.4	92.2

The trend of the inhibition of DPPH radical by the extract was concentration dependent with respect to the IC_{50} values (concentration of the extract to cause 50% inhibition), the DPPH radical scavenging ability of the extract showed the following trend methanol extract < Ascorbic acid. It is interesting to note that the lower the IC_{50} value, the higher the scavenging activity of the plant extract.

Table 3: Antioxidant Activity of the Methanol extract of *Combretum micranthum* leaves

Sample	IC_{50} (µg/mL)
Methanol	2.23
Ascorbic acid	0.042



The efficacy of crude methanol extract of *Combretum micranthum* leaves against *P. falciparum* indicated that *Combretum micranthum* significantly decreased the parasitic load at five different concentrations; 500, 250, 125, 62.5 and 31.25 mg/ml with percentage growth inhibitions of 88.8-66.6 %. Thus, from the results obtained, *Combretum micranthum* leaves extract is effective against *P. falciparum.*

Table 4. Anti-Malarial Activity of *Combretum micranthum* Leaves Extract against *P. falciparum* with Respect to their Growth Inhibition

Parameters	Concentration (mg/ml)					
	500	250	125	62.5	31.25	Control
Mean RBC	18	18	18	18	18	
Mean infected RBC	16	14.5	14	12.5	12.0	
Mean % Parasitaemia	2.0	3.5	4.0	5.5	6.0	
% Growth inhibition	88.8	80.5	77.7	69.4	66.6	-

Broad absorption band of hydroxyl occurred at 3188.8 cm^{-1} indicating the presence of –OH stretching depicting the presence of alcohols and phenols (hydrogen bonded), N-H stretching of amines. The FTIR Peaks obtained at 2925.8 cm^{-1} indicated the presence of C=O stretching of Carboxylic acids. Peak obtained at 2113.9 cm^{-1} indicated the presence of C≡N / C≡C stretching of aldehyde/amines/nitriles. 1774.9 and 1722.9 cm^{-1} showed C=O stretching of aldehyde/ketone/ester/amides and C=O/C=C stretching of aromatic hydrocarbons. Peak obtained at 1443.7 cm^{-1} indicated the presence of C-H/C=C bending of aromatic hydrocarbons.

Table 5. Peak positions and probable inter-atomic bond of isolated compounds from *Combretum micranthum* Methanol leaves extract by FTIR

IR	Functional group
3188.8	-OH Stretching of alcohol and phenol
2925.8	C-H stretching , C=O stretching of carboxylic acid, aldehyde and ketone
2113.94	C≡C stretching
1774.94	N-H bending of amines
1722.92	C=O stretching of amide, C=C
1443.79	C-N stretching of amines
1067.73	C-O stretching

The main organic compounds detected by GC-MS in methanol extract of *Combretum micranthum* is on (Tables 6). 13-Octadecanoic acid, Hexadecanoic acid, methyl stearate 9,12- Octadecanoic acid were found to be the major compounds detected in the leaves extract. In addition the leaves also contained oleic acid.

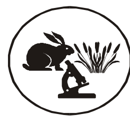


Table 6: Phytoconstituents identified in the Methanol extract of the *Combretum micranthum* Leaves by GC-MS

No. of Peaks	Retention time	% Area	Compound	Molecular weight	Structure	Molecular formula	MZ
1	14.20	8.84	Hexadecanoic acid (palmitic acid)	256.42	O, OH	$C_{16}H_{32}O_2$	256.24
2	16.34	1.34	Oleic acid	282.46	O, OH	$C_{18}H_{34}O_2$	282.26
3	18.23	8.09	Methyl stearate	298.50	O, O	$C_{19}H_{38}O_2$	298.29
4	20.45	8.65	9,12-octadecadienoic acid	280.45	O, OH	$C_{18}H_{32}O_2$	280.24
5	24.20	38.56	Cis-13-octadecenoic acid	282.46	O, OH	$C_{18}H_{32}O_2$	282.26
6	28.45	1.08	N-trifluoroacetylimidazole	164.09	O, F, F, F, N, N	$C_5H_3F_3NO_2$	164.02
7	30.12	3.89	n-propyl-11-octadecenoate	323.53	O, O^-	$C_{21}H_{39}O_2$	323.30

From the results of the acute toxicity studies (Table 7), no death was recorded in the first phase. In the second phase, doses of 1500, 2250, 3250 and 5000mg/kg were used and no death was also recorded. The oral median lethal dose (LD_{50}) for the methanol leaves extract of *Combretum micranthum* was therefore estimated to be greater than 5000mg/kg and no sign of behavioural changes were also observed.

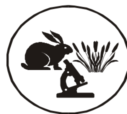


Table 7. Acute toxicity studies of methanolic extract of *Combretum micranthum* Leaves

Treatment	Group	Number of Animals	Dose (mg/kg)	Mortality recorded after 24hrs
Phase I	I	3	10	0/3
	II	3	100	0/3
	III	3	1000	0/3
Phase II	I	1	1500	0/1
	II	1	2250	0/1
	III	1	3250	0/1
	IV	1	5000	0/1

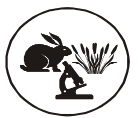
DISCUSSION

The natural plant products such as alkaloids and saponins have received greatest attention with regards to possible medicinal application. In addition, alkaloids and flavonoids were also reported to be responsible for antimicrobial properties in some ethno medicinal plants (Mohammed, 2013). Furthermore, many tannin molecules have also been shown to reduce the mutagenic activity of a number of mutagens. Many carcinogens and/or mutagens produce oxygen free radicals for interaction with cellular macromolecules. The anticarcinogenic and antimutagenic potentials of tannins may be related to their antioxidative property, which is important in protecting cellular oxidative damage including lipid per oxidation (Mohammed, 2013).

The remarkable antioxidant activities exhibited by methanol extract (Table 2) have made them potential free radicals scavenging agents, and this is probably due to their phenolic, alkaloids, flavonoids, terpenoids and vitamins constituents (Sri-sudewi *et al*., 2014). The facts mentioned above have proven the folkloric use of this plant in traditional medicine in Nigeria for the treatment of numerous diseases and health conditions such as malaria, fever, pains, diabetes and convulsion (Akuodor *et al.,* 2015; Akuodor *et al.,* 2013). The best antioxidant activities exhibited by methanol extracts is probably due to the presence of hydroxyl group attached to the aromatic ring of their molecules. This is supported by the report of Ahmad, (2011) where he stated that the mechanism of reduction of DPPH molecule by plant extracts or isolated pure compounds or fractions is due to the presence of hydroxyl groups on their molecules. This mechanism is possible in plants that are rich in flavonoids, tannins, anthocyanins, and anthocyanidins because they met the structural requirements.

The phytochemical study of methanol extract as performed by the FT-IR spectra has given many characteristic functional groups with various structural skeletons such as: amino acids, hydroxyl compounds, ethers, carbonyl compounds, carboxylic acid, aldehyde, ketone, amide (Mabozou *et al.,* 2019). These characteristic functional groups correspond to flavonoids, tannins, saponins, terpenoids, alkaloids, carbohydrate and steroids. One of the main goals of drug discovery is to identify molecules with antioxidant activity, which is often done through in vitro evaluation (Mabozou *et al.,* 2019).

The effect of antioxidants on DPPH radical scavenging is thought to be due to hydrogen donating ability. DPPH is a stable free radical and it accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Kannan *et al*., 2012). When these molecules are formed, the absorbance decreases and the DPPH solution decolourises from violet colour to pale yellow.

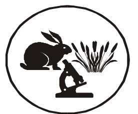


The degree of discolouration is an indication that the plant extract has the potential to scavenge free radicals as a result of its ability of hydrogen donation. More yellowish colour of DPPH is an indicator of stronger antioxidant activity of the extract (Sowunmi and Afolayan, 2015). The trend of the inhibition of DPPH radical by the extract was concentration dependent with respect to the IC_{50} values (concentration of the extract to cause 50% inhibition), the DPPH radical scavenging ability of the extract showed the following trend Ascorbic acid > extract. It is interesting to note that the lower the IC_{50} value, the higher the scavenging activity of the plant extract (Sowunmi and Afolayan, 2015). The DPPH test measured the hydrogen atom or electron donor capacity of the extract to the stable radical DPPH formed in solution (Orisakeye *et al.,* 2015). The activity was expressed as the concentration of sample necessary to give a 50% reduction in the sample absorbance (IC_{50}). The leaves extract exhibited considerable DPPH free radical scavenging activity as indicated by their IC_{50} values of 2.23 mg/ml (Orisakeye *et al.,* 2015).

In this study, leaves extract of *Combretum micranthum* was evaluated for their antimalarial activities by using an *in vitro* method. The findings revealed that the leaves extract showed inhibition of the formation of hemozoin (β-hematin), which suggests the presence of antimalarial compounds in the leaves. Samuel *et al.* (2014) in a separate study isolated a flavonoid from the acetone extract of *Combretum racemosum*. This correlate with some of the identified phytochemicals in some *Combretum* species as reported by Rodrigues *et al.* (2012). The presence of alkaloids in this plant species confirms the findings of Ajaiyeoba *et al.* (2006) that the traditional use of the plants for the treatment of malaria was due to the presence of alkaloids. Mfopa *et al.* (2017) in another study reported the presence of alkaloids in an extract with antimalarial activity. Alshawsh *et al.* (2007) reported that tannins may have antiplasmodial activity. The cardiac glycosides are used for treating heart problems that may result from severe malaria attack (Fatoba *et al.,* 2003).

From phytochemical screening reports, it was deduced that plants containing steroids play a role in the treatment of cerebral malaria, thus these steroids might have contributed to the antiplasmodial activity seen in *Terminalia ivorensis* and *Combretum racemosum* (David *et al.,* 2004). Tannins have also been found to be potentially anti-viral, anti-bacterial and anti-parasitic agents (Ene *et al.,* 2008). Dibua *et al.* (2013) also identified tannins, saponins, terpenoids, steroids, glycosides, proteins, fats and oil, and acidic compounds as possible antimalarial agents in medicinal plants. Also, Vargas *et al.* (2011) using the β-hematin model employed in a study tested the antimalarial activity of some flavonoids, alkaloids and tannins compounds, and found them to be very active comparable to the *in vitro Plasmodium falciparum* growth inhibition assay (NF54) used by several laboratories as the golden standard in the antimalarial drug screening. Thus, it is plausible to assume that all these secondary metabolites present in these plants could contribute to the high antimalarial activity observed and maybe also in other Combretaceae used traditionally for the treatment of malaria. The results of this study confirmed the traditional usage of some of these plants as reported in literature.

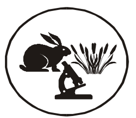
The FT-IR identified that the leaves extract contained strong C- H vibration for substituted benzene ring, indicating the presence of phenols and flavonoids in the crude *Combretum micranthum* extract. Flavonoids are polyphenols characterised by two benzene rings joined by a linear carbon chain (Pharmawati and Wrasiati, 2020).



The identification of benzenoid compounds via FT-IR spectrophotometry supported the findings from the phytochemical screening, which detected the presence of phenols and flavonoids. The amines, imines, alkanes and phenols present were considered the major functional groups of bioactive compounds (Pharmawati and Wrasiati, 2020).

Moreover, the medium peaks generated at 2925.8 cm^{-1} represent H-C=O: C-H stretching for aldehydes, with strong absorption peaks at 1722.92 cm^{-1} are assigned to C=O stretching vibration in carbonyl compounds; which may be characterized by the presence of high content for unsaturated aldehydes, esters and ethers (Maobe *et al.,* 2013). The peak around 1774.94-1722.92 cm^{-1} was assigned as C=O ester, which may be related to pheophytin and chlorophyll (Pharmawati and Wrasiati, 2020). In this study, the position of the peak was at 1774.94 cm^{-1} (Table 5). The carotenoids were predicted to be present in the dried leaf powder of *Combretum micranthum.* The FT-IR spectrum at 1722.92 cm^{-1} (Table 5) was assigned as the C=O conjugate. The conjugated double bond in carotenoids has been reported as the structure responsible for light absorption. A previous study stated that a peak at 1700 cm^{-1} is due to chlorophyll and protein content (Pharmawati and Wrasiati, 2020). Based on the phytochemical compounds discovered in the FT-IR analysis, *Combretum micranthum* has the potential to be used for biomedicinal applications, as phenolic compounds, tannins, flavonoids, chlorophyll and carotenoids are known to have antioxidant activities (Pharmawati and Wrasiati, 2020). These findings provide an opportunity for the development of natural products from *Combretum micranthum* in drug discovery. Interestingly, a number of peak values remained unidentified and would therefore require further spectroscopic analysis to determine the bond types they represent (Maobe *et al.,* 2013). They would then be isolated, identified, characterized and their molecular structures elucidated as they may be novel and responsible for the observed antimicrobial activities of *Combretum micranthum* leaves (Maobe *et al.,* 2013). Starlin *et al*. (2012) analyzed the ethanol extracts of *Ichnocarpus frutescens* using FTIR analysis that revealed functional group components of amino acids, amides, amines, carboxylic acid, carbonyl compounds, organic hydrocarbons and halogens. Nithyadevi and Sivakumar (2015) also worked in the methanol leaf extract of *Solanum torvum* to confirm the presence of alcohol, alkanes, aromatic carboxylic acid, halogen compound, alkyl halide through the FTIR analysis.

Gas chromatography study includes the important optimization process such as introduction of sample extract onto the GC column, separation of its components on an analytical column and detection of target analysis by using mass spectrometry (MS) detector. The spectrum of GC-MS analysis of methanol extract of the *Combretum micranthum* leaves revealed the presence of seven chemical compounds on the basis of retention times which were directly characteristic of certain compounds. Most of these separated chemical compounds had been reported for their pharmacological activities on the basis of their molecular formula and chemical structure that could contribute to the medicinal quality of the extract. Hexadecanoic acid, oleic and octadecanoic acids which may be involved in antagonism against bacteria (Al-Saif and Birnin-Yauri, 2013). These observed compounds have also been reported to have antioxidant, hypocholesterolemic, hemolytic, antiinflammatory, anticancer, antibacterial, nematicidal and antihypertensive activities (Nwodo *et al.,* 2015). These bioactive compounds that mainly comprised of carboxylic derivatives may be involved in the observed pharmacological activity.



The oral median lethal dose value for the methanol leaves extract of *Combretum micranthum* obtained in rats was found to be above 5000mg/kg. This suggests that the plant extract is non-toxic as no death was recorded. Acute toxicity studies are usually carried out to determine the dose that will cause death or serious toxic manifestations when administered singly or severally at few doses in order to establish doses that should be used in subsequent studies (Wanda *et al.,* 2002). The Organization for Economic Cooperation and Development (OECD), recommended the chemical labelling and classification of acute systemic toxicity based on oral median lethal dose values as: very toxic if ≤ 5 mg/kg, toxic if > 5 mg/kg but ≤ 50 mg/kg, harmful if > 50 mg/kg but ≤ 500 mg/kg, and non-toxic or not harmful if > 500 mg/kg or ≤ 2000 mg/kg (Walum, 1998). The LD_{50} was found to be greater than 5000 mg/kg when administered orally in rats (Table 7) and all the animals remained alive and did not manifest any significant visible signs of toxicity at these doses. This study showed that the extracts *Combretum micranthum* leaves are practically non-toxic when administered using the oral route. This is based on the toxicity classification which states that substances with LD_{50} values of 5000 to 15,000 mg/kg body weight are practically non-toxic (Loomis and Hayes, 1996).

CONCLUSION

The results of this study confirmed the presence of alkaloid, flavonoids, saponins, tannins, glycosides, phenols, triterpenes and steroids while anthraquinones were absent in the *Combretum micranthum* leaves extract. The DPPH radical scavenging ability of the extract showed the methanol extract has low antioxidant when compared with ascorbic acid.

The inhibitory effects of *Combretum micranthum* leaves crude extract against *P. falciparum* at concentrations of 500, 250, 125, 62.5 and 31.25 mg/ml with percentage growth inhibitions of 88.8-66.6 %. The presence of phytochemicals in this extract might have been responsible for the anti-malarial and antioxidant activities possessed by the plant, which supports the uses of this plant species to treat malaria associated symptoms in local/traditional settings.

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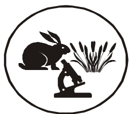

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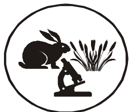

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