PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF THE LEAVES OF *COMBRETUM BAUCHIENSE* HUTCH & DALZIEL (COMBRETACEAE)

ABSTRACT

Aim: The phytochemicals, antioxidant and antimicrobial investigations of *Combretum bauchiense* leaves used in folkloric medicine to treat majorly infectious diseases were explored in the present study.

Study design: Evaluation of antioxidant, antimicrobial and HPLC-DAD profiling of the extract and fractions of the leaves of *Combretum bauchiense.*

Place and Duration of study: Department of Pharmaceutical Chemistry, Enugu State University of Science and Technology, Enugu, Nigeria between January 2021 and May 2023.

Methodology: The methanol extract (MECB), n-hexane fraction (HFCB), ethyl acetate fraction (EFCB) and n-butanol fraction of *C. bauchiense* (BFCB) were screened for phytoconstituents using HPLC-DAD. The antioxidant activity of the extract and fractions were assayed by DPPH free radical scavenging model while the antimicrobial activity of extract and fractions were carried out by agar well diffusion method.

Results: The following phytoconstituents: dukurolide, shanzinmethyl ester, piperchabamide, isovitexin, straitisporolide, kaempferol, apigenin, protocatechuic acid, aureonitol, luteolin, rutin and quercetin were identified from the extracts and fraction by dereplication based on their retention time and wavelength. The result of antioxidant assay indicated that MECB showed strong free radical scavenging activity with IC_{50} value of 3.22 µg/mL. Among the fractions, EFCB and BFCB have moderate free radical scavenging activity (IC_{50} value of 5.69 and 6.35 µg/mL respectively), compared to that of ascorbic acid (IC_{50} value of 0.21 µg/ mL). HFCB showed the least free radical scavenging activity (IC_{50} value of 10.78 µg/mL).

Conclusion: The extract and the fractions at the concentration of 6.25-100 mg/mL showed inhibition zone diameters, which ranged from 10-27 mm against all tested bacteria. The antioxidant and antimicrobial properties, exhibited by the extracts and fractions may be as a result of the presence of high concentrations of the phenolic compounds identified by HPLC-DAD analysis. The results further substantiated the folkloric uses of the plant in treating infections and some disease conditions associated with oxidative stress.

Keywords: *Combretum bauchiense; HPLC-DAD; Phytoconstituents; Antioxidant; Antimicrobial*

INTRODUCTION

The genus *Combretum* are well known in African traditional medicine, and all parts of the *Combretum* species are used as medicine in treating stomachache, wound healing, edema, skin diseases, diarrhea, muscle pain, and malaria [1-4]. The major compounds derived from the *Combretum genus* include but not limited to the flavonoids, terpenoids and stilbenoids as previously reported [5-8]. Several scientific work have been reported on the potentials of combretum species as anti-inflammatory, antimicrobial, antimalarial, hepatoprotective, antioxidants, anthelminthics and cytotoxicity [9-15].

Combretum bauchiense is one of the poorly investigated species of combretum. It is a suffrutex with short, erect, usually herbaceous stems arising from a woody root stock. It appears in savanna soon after fire and lowers within a few weeks. *The leaves are simple*, opposite, whorled, sub opposite or alternate, *almost always entire [16].* The plant is traditionally used in treating feverish condition, infectious diseases and as tonic. The biological activities of *C. bauchiense* reported so far are antidiarrheal, analgesic and antipyretic and antibacterial [17-19]. The preliminary phytochemical investigation revealed the presence of flavonoids, tannins, cardiac glycosides, terpenoids, and saponins. From the previous studies on the other species of combretum, they demonstrated a significant biological activities due to presence of bioactive compounds. This study was undertaken because of paucity of information on the phytochemicals and pharmacological properties of *Combretum bauchiense*. Therefore, we investigated for the first time the antimicrobial and antioxidant activities of extract and fractions of *Combretum bacuhiense* leaves and employed HPLC-DAD analysis for the online detection and identification of the bioactive constituents.

MATERIALS AND METHODS

COLLECTION AND PREPARATION OF PLANT MATERIAL

The leaves of *Combretum bauchiense* were collected in Nsukka, Enugu State, Nigeria on the 23rd of January 2021. The plant was authenticated by Mr Felix Nwafor of Department of Pharmacognosy and Environmental Medicine University of Nigeria, Nsukka, Enugu State, Nigeria. A voucher specimen has been deposited at the herbarium (herbarium number 019/02). The leaves were washed on tap water, air dried for 14 days. The air dried leaves were pulverized using a laboratory scale slow speed electric blender to obtain a homogenous sample which was used for the extraction

CHEMICALS AND EQUIPMENT

Analytical grades of methanol, n-hexane, ethyl acetate and n-butanol (Honeywell, Germany), ascorbic acid (Qualikems), DPPH (Sigma Aldrich Germany) Levofloxacin (Kinvox), ketoconazole capsules (Greenlife), Nutrient agar (by TM Media United Kingdom), Sabouraud Dextrose Aga (by TM Media United Kingdom), Isopropyl alcohol (Moko Methylated spirit), distilled Water were used. HPLC Methanol (LiChroSolv HPLC; Merck) and nano-pure water (distilled and heavy metals free water obtained by passing distilled water through nano- and ion-exchange filter cells; Barnstead, France). All other chemicals used were of AnalaR grade. Concentration of extract and fractions were done in rotary evaporator (Digital) TT-52 (by Techmel and Techmel, USA), Pressure cooker autoclave (by Bondtech Corporation, USA), incubator (by Genlab), and UV-visible spectrophotometer (6505, Jenway, England).

MICROORGANISMS

The microorganisms used in this research work are pathogenic in nature and were obtained from few different patients that visited Adonai Biomedical and Microbiological Laboratory and Research Centre, Nsukka. The test organisms were six in numbers comprising of two (2) gram positive bacteria, three (3) gram negative bacteria and one fungus. These organisms includes: *Staphylococcus aureus* and *Streptococcus pneumoniae*; *Salmonella typhi*, *Escherichia coli* and *Klebsiella pneumoniae*; and *Candida albican*. These microbial specimens were cultured and their pure isolates were obtained.

EXTRACTION AND FRACTIONATION

The pulverized plant sample weighing 1kg was macerated in methanol (7 L) for 72 hours with intermittent agitation. Thereafter the mixture was filtered, concentrated using rotary evaporator under reduced pressure, to obtain the methanol extract of *Combretum bauchiense* (MECB). The MECB weighing 100g was dispersed in 200 mL of water containing 20% methanol. The reconstituted solution was subjected to liquid-liquid extraction for fractionation based on solvent polarity using (n-hexane; 500 mL x 3), (ethyl acetate; 500 mL x 4) and (n-butanol; 300 mL x 2). The fractions were concentrated using rotary evaporator under reduced pressure to afford hexane fraction of *Combretum bauchiense* (HFCB), ethyl acetate fraction of *Combretum bauchiense* (EFCB) and buthanol fraction of *Combretum bauchiense* (BFCB) respectively.

HPLC-DAD PROFILING OF EXTRACT AND FRACTIONS

Each of the dried crude extract and fractions (2 mg) was dissolved 2 mL of HPLC grade methanol and the mixture was centrifuged at 3000 rpm for 5 min. Then, 100 µL of the dissolved samples was transferred into HPLC vials containing 500 µL of HPLC grade methanol. HPLC analysis was carried out on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector. Detection was at 235, 254, 280, and 340 nm. The separation column (125 mm × 4 mm; length × internal diameter) was prefilled with Eurosphere C- 18 (Knauer, Germany), and a linear gradient of nanopure water, adjusted to pH 2 by addition of formic acid and methanol was used as the eluent. Compounds were detected using diode array and identified based on similarity with data in the inbuilt library.

DPPH FREE RADICAL SCAVENGING ACTIVITY OF EXTRACT AND FRACTIONS

The antioxidant activity of crude extract and fraction of *C. bauchiense* were determined using the 1, 1-diphenyl- 2-picrylhydrazyl (DPPH) free radical scavenging assay by previously described method [20]. A stock solution 300 µg/mL of extract and fractions were prepared in methanol and test samples were prepared from stock solution by dilution with methanol to obtain different concentrations ranging from

18.75 µg/mL to 150 µg /mL. DPPH solution was prepared in methanol. A 1 mL of DPPH solution (0.6 mM) was added to 1 mL of different concentrations of the extract and fractions. The volume of the solution was adjusted with methanol to a final volume of 5 mL. The mixture was incubated in the dark for 30 min at room temperature and absorbance of the mixtures was obtained at 517 nm using VIS spectrophotometer (6505, Jenway, England). Ascorbic acid was used as standard. The absorbance of the control containing 1 mL of DPPH solution and 4 mL of methanol was used to calculate the free radical scavenging activities. The percentage free radical scavenging potentials of the extracts, fractions and standard (ascorbic acid) were calculated using the equation below.

Agar diffusion method

The agar well diffusion method previously reported was used [21]. The extract and fractions were dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 100 mg/mL for each of the test agents. From the 100 mg/mL stock solutions of each of the test agents, two (2) fold serial dilutions were done to obtain concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL. 0.1mL of 0.5McFarland standard of the test microbial cells suspensions was placed in the petri-dish and 20 mL of the moderately warmed sterilized nutrient agar was added and the mixture was mixed properly by rotating the plate in a circular manner on the working bench for some time to ensure appropriate admixture. This was then allowed to seed at room temperature. Wells of about 5 mm in diameter were made in the seeded agar plate using a 5 mm sterile cork-borer. These wells were filled with 50 µL of the different dilutions of the test solutions using a sterilized microliter pipette. These procedures were followed using 10µg/mL of Levofloxacin as the positive control agent against the test organisms. A triplicate of each of these was obtained. These microbial cultures were then incubated for 24 h at 37ºC for bacteria and 24ºC for fungi. After the incubation period, the respective inhibition zone diameters around each well were measured in millimeters.

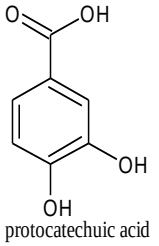
RESULTS

The HPLC-DAD analysis of extract and fractions indicated the presence of bioactive compounds with known pharmacological properties. Most of the bioactive compounds identified in the *C. bauchiense* leaves are moderately polar compounds: flavonoids, terpenoids, fatty acid and their derivatives as shown in Table 1-4 with their respective retention time, molecular weight, molecular formula and reported biological activity.

Table 1: Phytocompounds identified in the MECB

s/n	RT min	Name of compound	Mol. weight	Molecular formula
1	4.23	Protocatechuic acid	154.12	$C_7 H_6 O_4$
2	6.62	Piperchabamide	411.23	$C_{26} H_{37} N O_3$
3	14.03	Pestalotioprolide	266.33	$C_{15} H_{22} O_4$
4	15.14	4-O-demethylisobutyrolactone	382.5	$C_{22} H_{38} O_5$
5	16.98	Neobavaisoflavone	322.4	$C_{20} H_{18} O_4$
6	18.30	Kaemferol-3-o-gall	594.5	$C_{27} H_{30} O_{15}$
7	14.25	Rutin	610.5	$C_{27} H_{30} O_{16}$
8	19.32	Luteolin	286.24	$C_{15} H_{10} O_6$

9	21.29	Eriodictiol	288.25	$C_{15}H_{12}O_6$
10	22.08	Quertcetin	302.24	$C_{15}H_{10}O_7$
11	25.28	Kaempferol	286.23	$C_{15}H_{10}O_6$
12	30.09	Apigenin	270.05	$C_{15}H_{10}O_5$
13	31.26	Aloeresin A	540.6	$C_{28}H_{28}O_{11}$
14	33.72	Dukunolide F	484.5	$C_{26}H_{28}O_9$
15	34.05	Expansol B	464.6	$C_{29}H_{36}O_5$
16	35.96	Benzylpyridine	169.22	$C_{12}H_{11}N$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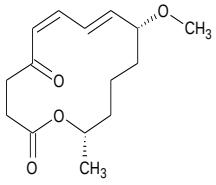
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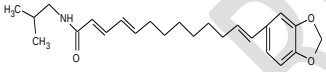
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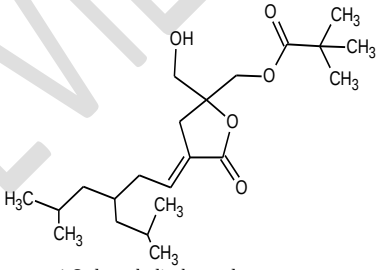
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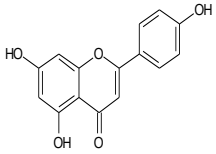
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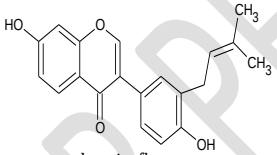
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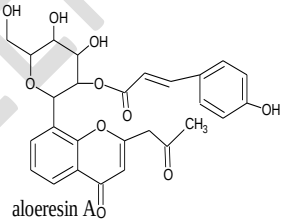
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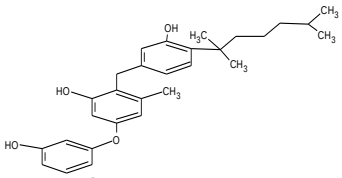
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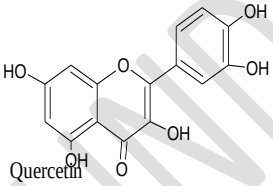
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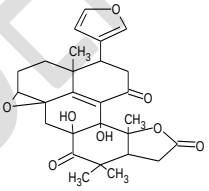
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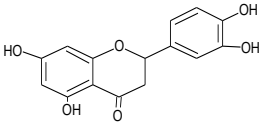
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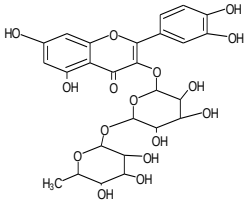
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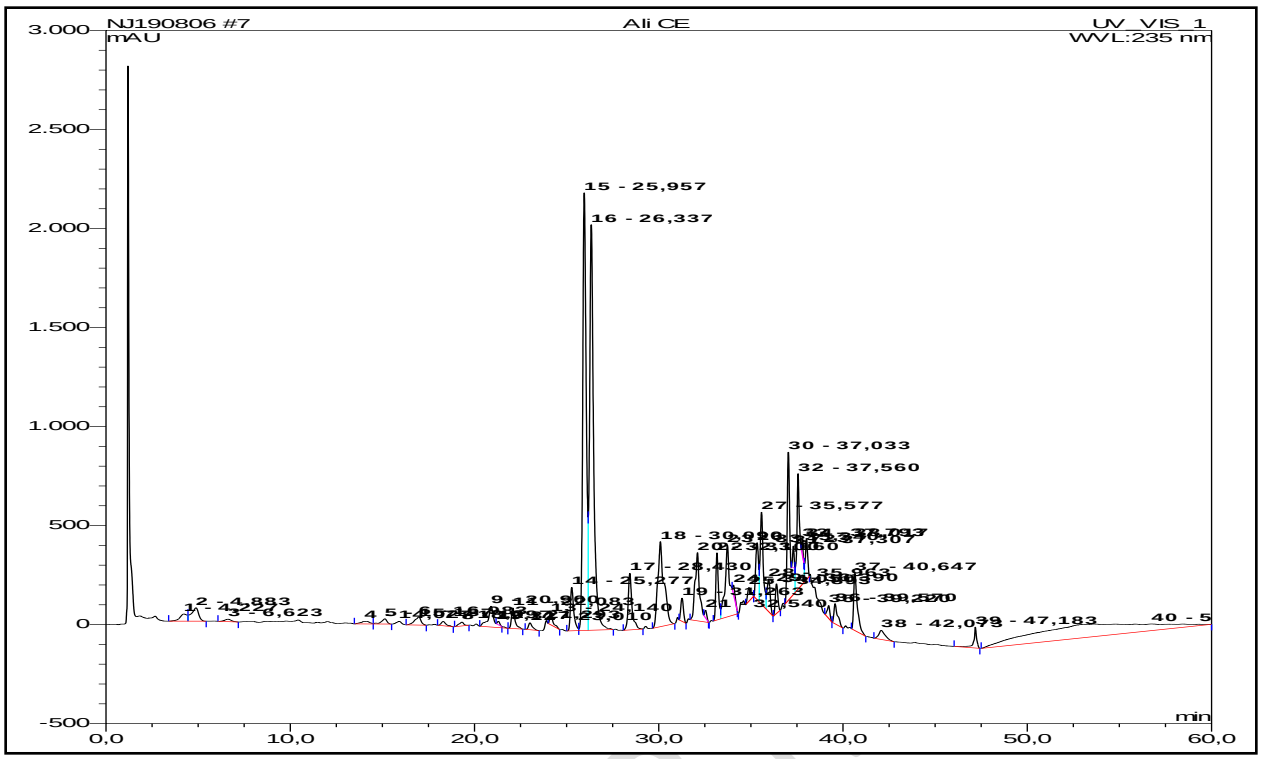
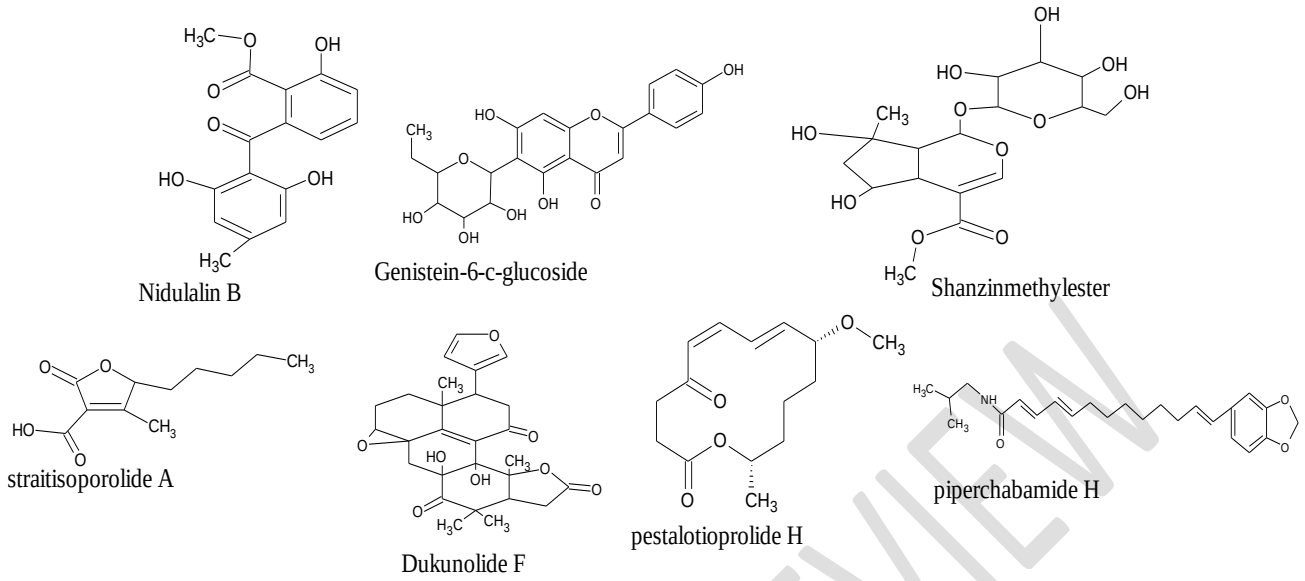


Figure 1: Chemical structures of pytocompounds identified in MECB

Table 2 Phytocompounds identified in HFCB

s/n	RT min	Name of compound	Molecular weight	Molecular formula
1	34.90	Striatisporolide	212.25	C_{11} H_{16} O_4
2	35.57	Dukunolide F	484.5	C_{26} H_{28} O_9
3	35.96	Piperchabamide H	411.23	C_{26} H_{37} N O_3
4	36.39	Genistein -6-c-glucoside	432.4	C_{21} H_{20} O_{10}
5	37.05	Shanzinmethyl ester	406.15	C_{17} H_{16} O_{11}
6	37.54	Pestalotioprolide H	266.33	C_{15} H_{22} O_4
7	39.58	Nidulalin B	302.28	C_{16} H_{14} O_6



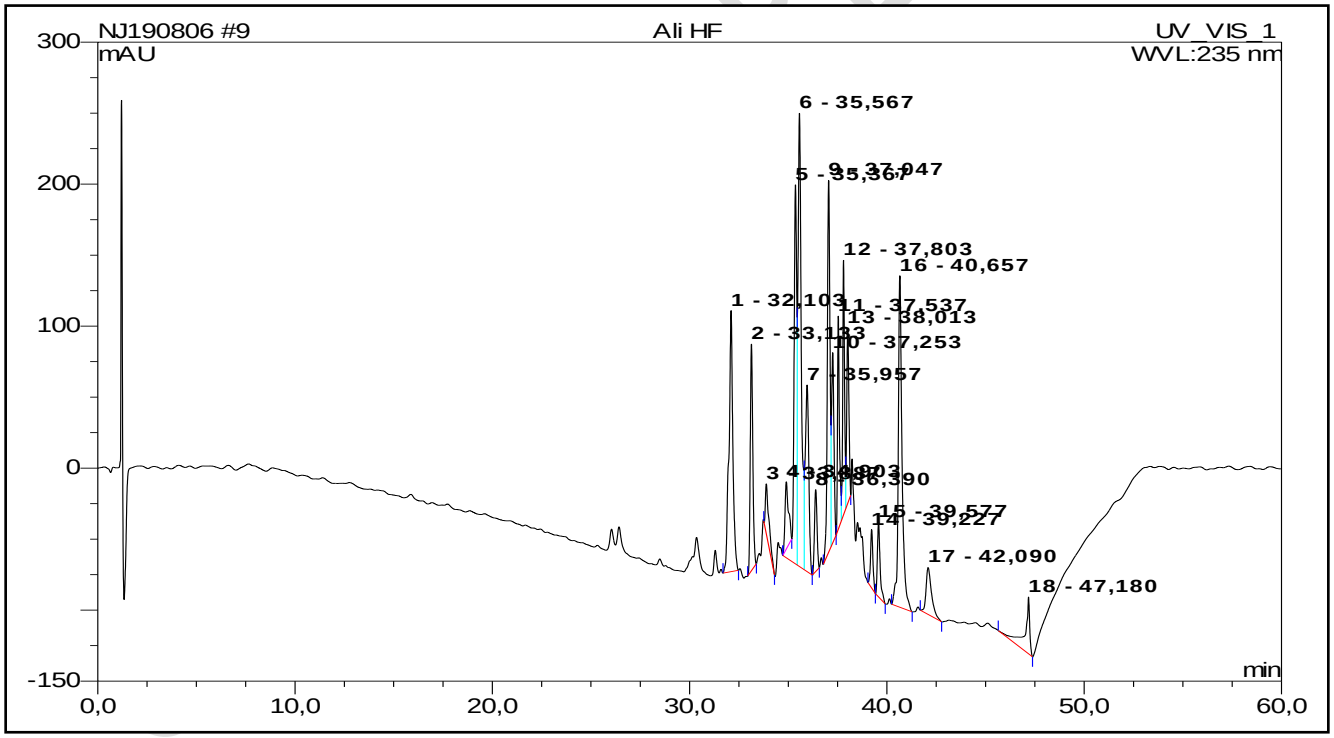
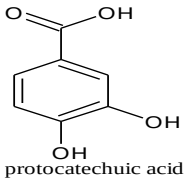


Figure 2: Chemical structures of phytocompounds identified in HFCB

Table 3: Phytocompounds identified in EFCB

s/n	RT min	Name of compound	Molecular	Molecular formula

			weight	
1	4.85	Protocatechuic acid	154.12	$C_7 H_6 O_4$
2	16.80	Aplysinamisin-2	650.2	$C_{23} H_{28} Br_3 N_3 O_4$
3	22.11	Eriodictiol	288.25	$C_{15} H_{12} O_6$
4	24.20	Luteolin	286.24	$C_{15} H_{10} O_6$
5	25.34	Kaemferol	286.23	$C_{15} H_{10} O_6$
6	26.03	Isovitexin	432.38	$C_{21} H_{20} O_{10}$
7	30.15	Apigenin	270.05	$C_{15} H_{10} O_5$
8	31.32	Aloeresin A	540.6	$C_{28} H_{28} O_{11}$
9	32.14	Aureonitol	206.28	$C_{13} H_{18} O_2$
10	35.90	Benzylpyridine	169.22	$C_{12} H_{11} N$
11	37.04	Shanzinmrthyl ester	406.15	$C_{17} H_{16} O_{11}$
12	37.57	Striatisporolide A	212.25	$C_{11} H_{16} O_4$
13	40.64	Nidulalin B	302.28	$C_{16} H_{14} O_6$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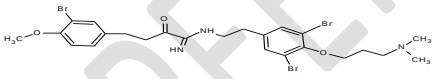
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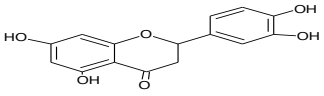
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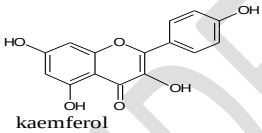
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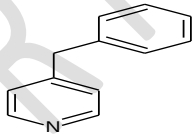
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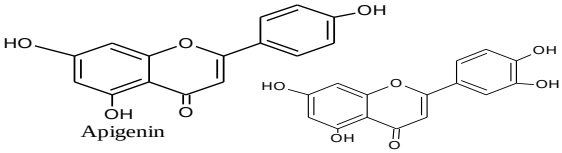
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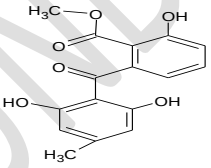
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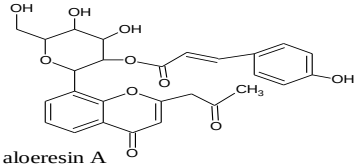
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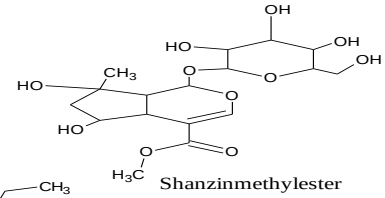
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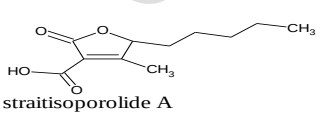
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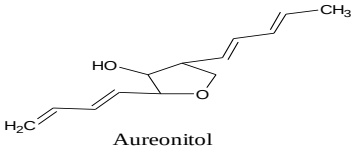
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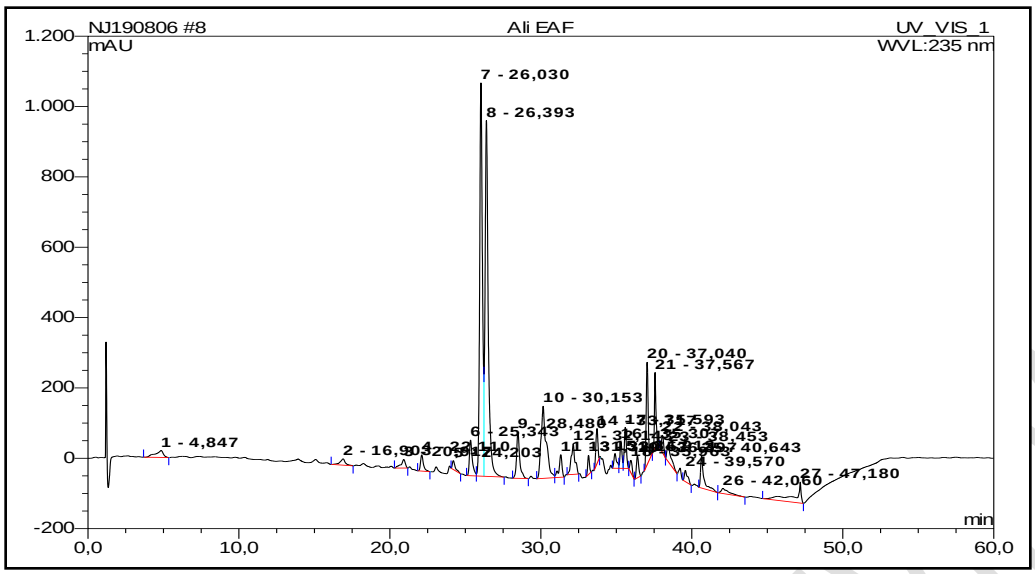

Figure 3: Chemical structures of phytocompounds identified in EFCB

Table 4: phytocompounds identified in BFCB

s/n	RT min	Name of compound	Molecular weight	Molecular formula
1	14.77	Cyclo(tyrosylprolyl)	260.28	C_{14} H_{16} N_2 O_3
2	19.27	Rutin	610.5	C_{27} H_{30} O_{16}
3	20.93	Apigenin	270.05	C_{15} H_{10} O_5
4	37.37	Hydroxyanthranilinic acid	153.14	C_7 H_7 N O_3
5	38.47	Oleic acid	282.5	C_{18} H_{34} O_2
6	47.18	Cyclopenol	310.3	C_{17} H_{14} N_2 O_4

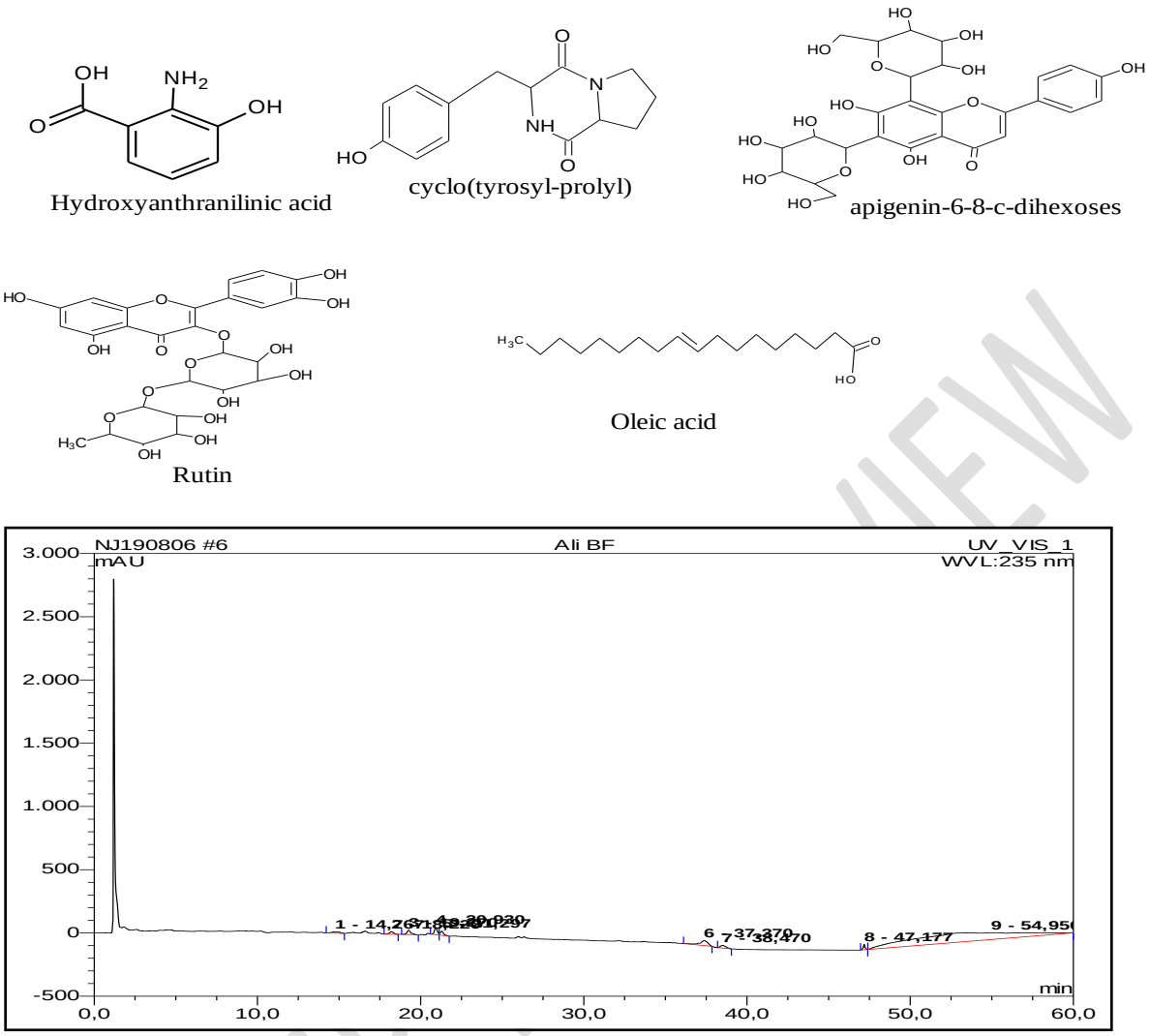

Figure 4: Chemical structures of phytocompounds identified in BFCB

DPPH FREE RADICAL SCAVENGING CAPACITY DETERMINATION

The antioxidant potential of extract and fractions were determined by DPPH free radical savenging activity. The extract demostrated good free radical scavenging activity more than any of the fractions and to an extent comparable to the ascorbic acid (standard). The extract had, inhibition of 76.9% at 300 µg/mL while different solvent fractions showed percentage inhibitions of 31.8% at 300 µg/mL for HFCB, 46.3% at 300 µg/mL for EFCB and 47.41% at 300 µg/mL for BFCB respectively. The ascorbic acid indicated percentage inhibition of 91.8% at 300 µg/mL. The antioxidant capacity of the extract and fractions also expressed as IC_{50}, which is the concentration that showed 50% inhibition of the free radical (Table 5). The result indicated that MECB had the lowest IC_{50} value of 3.22 µg/mL showing good free radical scavenging activity. However, among the fraction EFCB and BFCB have significant free radical scavenging activity (IC_{50} value of 5.69 and 6.35 µg/mL respectively). HFCB showed the least free radical scavenging activity (IC_{50} value of 10.78 µg/mL).

Table 5: IC_{50} of the extracts and fractions

Samples	IC_{50} Values (µg/mL)
MECB	3.22
HFCB	10.78
EFCB	5.69
BFCB	6.35
AA	0.21

KEY: methanol extract of *C. bauchiense* (MECB), n-hexane fraction of *C. bauchiense* (HFCB), ethyl acetate fraction *of C. bauchiense* (EFCB), n-butanol fraction of *C. bauchiense* (BFCB) and ascorbic acid (AA)

ANTIMICROBIAL EVALUATION

The results of the antimicrobial sensitivity test of MECB, HFCB, EFAO and BFCB are shown in Table 6. The extract and the fractions at the concentration of 6.25-100 mg/mL showed inhibition zone diameters, which ranged 10-27 mm against all tested bacteria. The HFCB and EFAO had inhibition zone diameter ranged 10-25 mm against all tested bacteria, at concentration range of 6.25-100 mg/mL following 2-fold serial dilution. The BFCB exhibited inhibition zone diameter of 13-19 mm against only *S. aureus* at 6.25-25 mg/mL whereas 50 -100 mg/mL showed inhibition zone of 10-13 mm against *S. typhi, K pneumonia*, and *E coli.* The standard drugs had inhibition zone diameter, which ranged from 27-45 mm at concentration of 0.01 µg/mL.

Table 6: Inhibition zone diameter (mm) of MECB, HFCB, EFCB and BFCB against test organisms

Samples	Conc. mg/Ml	IZD (mm)					
		Staphylococcus aureus	*Salmonella typhi*	Klebsiella *pneumonia*	*Escherichia coli*	*Streptococcus pneumonia*	*Candida albican*
MECB	100	27 ± 0.82	25 ± 1.41	25 ± 0.74	24 ± 0.82	26 ± 0.47	0
	50	24 ± 0.82	23 ± 0.82	21 ± 0.82	24 ± 0.82	23 ± 0.47	0
	25	20 ± 0	20 ± 0	21 ± 0.82	19 ± 1.63	19 ± 0	0
	12.5	20 ± 0.82	17 ± 0.82	18 ± 0	17 ± 0.82	16 ± 0.82	0
	6.25	17 ± 0.	15 ± 0.82	15 ± 0	14 ± 0	12 ± 0	0
HFCB	100	21 ± 0	19 ± 0.47	20 ± 0	20 ± 0	22 ± 0.82	0
	50	18 ± 0	17 ± 0	16 ± 0.82	19 ± 0.82	19 ± 0	0
	25	16 ± 0.82	15 ± 0	15 ± 0	19 ± 0.82	17 ± 0	0
	12.5	15 ± 0.82	14 ± 0	13 ± 0	17 ± 0.82	14 ± 0	0
	6.25	13 ± 0	12 ± 0.82	12 ± 0	13± 0.82	14 ± 0	0
EFCB	100	19 ± 0.82	18 ± 0.82	17 ± 0	20 ± 0	23 ± 0.82	0
	50	18 ± 0	16 ± 0	15 ± 0	18 ± 0.82	21 ± 0	0
	25	15 ± 0	14 ± 0.82	13 ± 0	16 ± 0	19 ± 0	0
	12.5	14 ± 0.82	14 ± 0	12 ± 0	14± 0	17 ± 0	0
	6.25	12 ± 0	12 ± 0	11 ± 0	10 ± 0	14 ± 0	0
BFCB	100	13 ± 0	16 ± 0	14 ± 0	13 ± 0	0	0
	50	10 ± 0	13 ± 0	10 ± 0	10 ± 0	0	0
	25	19 ± 0	0	0	0	0	0
	12.5	15 ± 0	0	0	0	0	0
	6.25	13 ± 0	0	0	0	0	0
LEVO	0.01	32 ± 0.05	32 ± 0.05	32 ± 0.05	32 ± 0.05	32 ± 0.5	-
KETO	0.01	-	-	-	-	-	27 ± 034

Key: methanol extract of *C. bauchiense* (MECB), n-hexane fraction of *C. bauchiense* (HFCB), ethyl acetate fraction *of C. bauchiense* (EFCB) , n-butanol fraction of *C. bauchiense* (BFCB). LEVO (levofloxacin) and KETO (ketoconazole).

DISCUSSION

The HPLC-DAD analysis of the extract (MECB) and the fractions (HFCB, EFCB &BFCB) of *C. bauchiense leaves* detected a whole range of bioactive compounds, which are majorly of the flavonoids (Tables 1-4; Figures 1-4). The major components identified by dereplication include luteolin, quercetin, kaempferol, apigenin, isovitexin and rutin, which have been shown to demonstrated significant pharmacological activities in previous studies [22-23]. These included but not limited to antimicrobial, antioxidant, anticancer, anti-inflammatory and antidiabetic activities [24-35]. The extracts and fractions demonstrated a dose dependent inhibition of the DPPH free radicals *in vitro* (Table 5). Our results indicated that EFCB, which showed the best antioxidant activity when compared with the other fractions had the highest concentration of phenolic compounds, mainly flavonoids (Table 3, Figure 3). It has been reported severally that flavonoids class of phytoconstituent possesses very good antioxidant properties [36-37]. Its antioxidant activity is due to presence of many phenolic hydroxyl groups in the molecule. These phenolic hydroxyl groups, particularly when they are in ortho-position have been shown to have immense ability to scavenge free radicals, chelate metal ions and inhibit lipid peroxidation [38]. The strong antioxidant activity of this plant may be associated with the high concentration of these phenolic compounds and this further justifies the use of the plant in the management of diseases associated with oxidative stress.

The preponderance of multi-drug resistant pathogens poses a serious threat to human and animal health. These microbial pathogens continue to develop intrinsic resistance to the available antibiotics [39]. The strategies could be by drug-degrading and -modifying enzymes, reduced membrane permeability for drugs, expression of drug efflux pumps, overproduction and alteration of drug targets, and bypassing the inhibitory pathway [39]. There is thus an urgent need to discover new antimicrobial agents with novel mechanism of action, to combat the multidrug resistance organisms. Our current study showed that the extract and fractions from *Combretum bauchiense* leaves possess broad spectrum antibacterial action but no antifungal property (Table 6). This is in agreement with previously reported antimicrobial activity of the other plants of *Combretum species* [40-41]. The broad spectrum antibacterial activity can also be attributed to the presence of the phenolic compounds in the plant. Flavonoids and other phenolic compounds have been severally reported to exhibit strong antimicrobial activity against resistance pathogens [42-43].

CONCLUSION

The result obtained demonstrated that leaves of *C. bauchiense* possesses antioxidant and antimicrobial properties, which may be as a result of the presence of copious concentrations of the phytoconstituents identified by HPLC-DAD analysis. Our findings also further validated the ethnomedicinal use of the leaves of *C. bauchiense* in the management of infections and various disease affecting man.

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