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Cytotoxicity, Antimicrobial and Antioxidant Activities of Apocarotenoid Rich Leaf Essential Oil of *Maesobotrya Barteri Var. Barteri* (Baill.) Hutch

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Abstract

Background: *Maesobotrya barteri var. barteri* (*Phyllanthaceae*) has analgesic, antimicrobial and antitussive applications in ethno-medicine. Essential oils and their constituents from herbs have also been utilized in the management of a good number of ailments in ethno-medicine. The phytochemical profile along with the corresponding cytotoxic, antimicrobial and antioxidant activities of the leaf essential oil have been investigated in the current research. The essential oil was obtained by hydro-distillation using an all-glass Clevenger apparatus. Identification and characterization were done using Gas Chromatography-Mass Spectrometry (GC/MS) while cytotoxicity assay was carried out by brine shrimp lethality test. The antimicrobial property was assessed by broth dilution method and antioxidant activity was evaluated with 2, 2-diphenyl-1 picrylhydrazyl radical (DPPH*) method.

Results: The essential oil contained a total of twenty-six compounds constituting 91.50 % of the total oil composition dominated with apocarotenoids comprising 49.4%, of which (E)-geranyl acetone (21.9 %) is the most abundant. Some other apocarotenoid constituents of the essential oil include β -ionone (8.9 %), 4-dehydro-β-ionone (5.4 %) and (E)-β-ionone (5.0 %). Brine shrimp lethality test revealed that the essential oil was non-toxic. The essential oil exhibited significant inhibition against bacterial and fungal strains at concentrations ranging from 1000 µg/mL to 62.5 µg/mL. *M. barteri* leaf essential oil exhibited significant antioxidant activity (% inhibition 90.04-84.73µg/mL) comparable to standard Ascorbic acid ((93.05- 90.40µg/mL) and butylated hydroxy anisole (92.60 - 90.68µg/mL) used for the assay.

Conclusion: The chemical composition of leaf essential oil of *M. barteri* could be responsible for the significant pharmacological applications of the plant in ethno-medicine and the phytoconstituent of the leaves essential oil of *M. barteri* is reported for the first.

Keywords: *Maesobotrya barteri*; Cytotoxicity; Antioxidant; antimicrobial; essential oil; apocarotenoids; (E)-geranyl acetone

Abbreviations: GC/MS: Gas Chromatography/Mass Spectrometry; FRIN: Forestry Research Institute of Nigeria; DMSO: Dimethyl sulfoxide; IC_{50}: The half-maximal inhibitory concentration; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; LRI: Linear Retention Index; TIC: Total ion concentration in percentage; GC: Gas Chromatography; ANOVA: Analysis of variance; NIST: National Institute of Standards and Technology; MBL: *M. barteri* leaf; BHA: Butyl hydroxyanisole; DMBA: 7,12-dimethylbenz(a)anthracene

Background

Essential oils and their constituents since ancient times have been known for their oral, topical, aromatherapy properties and their wide range of applications in consumer goods such as soaps, detergents, cosmetics, pharmaceuticals, confectionery, perfumes, soft drinks, beverages and insecticides are due to their therapeutic importance and lower toxicity profile [1]. Its phytoconstituents play an important role as an ideal natural source for antimicrobial, antioxidant, anticancer and chemo preventive agents [2-6].

Maesobotrya barteri var. bateri (Baill.) Hutch (*Phyllanthaceae*) is a high forest flowering shrub commonly found in the rainforest region of West Africa and the most widely distributed *Maesobotrya* species in Nigeria [7,8]. Its fruits are edible, succulent and other parts are used for the treatment of diarrhea, dysentery, urethral discharge, cough and pain reliever [9,10]. Phytochemicals such as tannins, saponins, cardiac glycosides, deoxy sugar and terpenes have been reported in its leaf, stem bark and root extracts [11,12]. Its pharmacological relevance includes analgesic, antimalarial [13] and antimicrobial [12] activities. β-amyrin and oleanolic acid isolated from the aerial part of M. barteri have been reported to exhibit antibacterial activity [14]. However, there is little or no report on the essential oil composition of *M. barteri*

leaf in the literature notwithstanding its medicinal and nutritional importance in ethnomedicine. This has prompted us to undertake this study and we herein report the GC/MS analyses, antioxidant, antimicrobial and cytotoxicity activities of *M. barteri* leaf essential oil.

Methods

Plant Collection and Preparation

Fresh leaves of *Maesobotrya barteri* were collected along an open area in Utit Uruan, Akwa Ibom - Nigeria, in October 2018. The fresh leaves were identified and authenticated at Forest Research Institute of Nigeria (FRIN), Oyo State, Nigeria where a voucher specimen with herbarium number FHI 112780 was deposited in the FRIN herbarium.

Chemicals and reagents

n-Hexance and methanol were purchased from BDH general purpose chemicals and distilled prior to use. Dimethylsulphoxide (May & Bayer), England and 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, butylated hydroxyanisole (BHA), Gentamicin and Tioconazole were obtained from Sigma Chemical Co. (St. Louis, MO).

Equipment and Apparatus

All-glass Clevenger, UV-Visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models) and GC-MS (Agilent 7809A gas chromatography hyphenated with an Agilent mass detector).

Isolation of Essential Oils

Fresh Leaf of *M. barteri* (200.01g) were placed in an all-glass Clevenger-type apparatus according to British Pharmacopoeia specifications [15] and hydro-distilled for 3hrs. Essential oil (MBL) was collected using *n*-hexane and dried over anhydrous sodium sulfate, filtered and stored in a refrigerator at 4°C prior to analysis. The yield of the oil was calculated on a dry basis.

Identification and Quantification of the Essential Oil Constituents

The leaf essential oil of *M. barteri* were subjected to GC-MS analysis on an Agilent 7809A gas chromatograph hyphenated with an Agilent mass detector having split/split less injector interfaced to mass selective detector operating at 70 eV. Other experimental parameters were as follows: The ion source temperature was set at 200ºC within a mass spectral range of m/z 50-700 at a scan rate of 1428 amu/sec. GC column was equipped with an HP-5MS column with a length of 30 m, having an internal diameter of 0.25mm and a film thickness of 0.25 µm. The oven temperature was programmed as follows: initial temperature of 80ºC for 2min, increased at 10ºC/min to the temperature of 240ºC for 6min.

Helium was utilized as the carrier gas at a flow rate of 1 mL/min. Injection volume, linear velocity and pressure were respectively adjusted at 1.0 µL, 362 cm/s and 56.2 KPa. The oven temperature was set at 60ºC, held for 1 min to 180ºC for 3 min at 10ºC /min, the final temperature was 280ºC for 2 min at 10ºC/min. Both injector and detector temperatures were fixed at 250ºC. Identification of the essential oil components was based on their linear retention indices as determined according to homologous series of normal alkane as well as by comparison of their mass spectral fragmentation patterns (NIST data/base/chemstation data system) with the data previously reported in the literature [16].

Brine Shrimp Lethality Test

Brine shrimp lethality test of the oil was conducted as described below: About 40 mg of shrimp's eggs were sprinkled on shallow rectangular plastic into a larger compartment of two unequal compartments (70/30) containing seawater. The shrimp's eggs were incubated for 48hrs in dark at room temperature. The shrimps (*nauplii*) were harvested on the smaller compartment exposed to a light source. The essential oil was dissolved in DMSO, and different concentrations were prepared using the serial dilution method (1000, 100 and 10 µg/mL). Ten *nauplii* were introduced in vials containing different concentrations of the oil. DMSO was tested as solvent control while doxorubicin was used as a reference standard. After 24hrs, the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined [17]. Each experiment was carried out in triplicate and the IC_{50} (concentrations at which 50 % of *nauplii* were killed) were calculated from inhibition versus concentration graphs using Microsoft Excel package.

Antimicrobial Activity on the Leaf Essential Oil of *M. Barteri*

All microorganisms were clinical isolates obtained from the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria. Leaf essential oil of *M. barteri* was tested against seven strains of microorganisms consisting of four bacteria; two Gram-negative; *Escherichia coli* and *Klebsiella pneumonia*, two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and three fungi (*Candida albicans*, *Penicillium notatum* and *Aspergillus niger*). Pour broth dilutions and surface plate methods were employed for antibacterial and antifungal activities, respectively [18]. The oil sample was prepared such that 1mL of the oil was regarded as 100 % concentration; 0.5mL into 0.5mL of DMSO to give 50 % concentration and 25 % concentration was obtained using serial dilution. DMSO was tested as solvent control, Gentamicin (10 µg/mL) as bacteria control and Tioconazole (0.7 mg/mL) as fungi control. All experiments were carried out in duplicates and observed zones of inhibition of growth were measured and recorded in millimeters (mm).

Antioxidant Activity of *M. Barteri* Leaf Essential Oil

Free radical scavenging activities of the essential oil and standards were determined by their ability to react with 2,2-diphe-

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nyl-1-picrylhydrazyl radical (DPPH*) [15,19]. A methanol-DPPH* solution (0.1 mM) was prepared by dissolving 3.94 mg of DPPH* in 100 mL of methanol. The oil was dissolved in methanol to prepare five concentrations (1000, 500, 250, 125 and 62.5) µg/mL via serial dilution and was mixed with 2.0mL of DPPH* methanol solution (0.1 mM). The mixture was shaken robustly and left to incubate for 30 mins in the dark at room temperature. The absorbance at 517nm was recorded for sample using a GS UV-12, UV-Vis spectrophotometer. A control experiment was carried out applying the same procedure but without essential oil (DPPH* + Methanol) and the absorbance was recorded as A_c. The antioxidant activity of ascorbic acid and butylated hydroxy anisole (BHA) were used as standards for comparison. Each experiment was carried out in triplicates and the free radical scavenging activity of the essential oil was calculated as percentage inhibition (%I) using the formula:

$$\%I = \frac{Acontrol - Asample}{Acontral} \times 100$$

Data Analysis

All results were conveyed as means ± standard deviation. Analysis of variance was used to determine any significant difference between groups using the statistical analysis software package GraphPad 5.0. Values with $p<0.05$ were regarded as significant. All graphs/charts were drawn with GraphPad 5.0.

Results

Phytochemical Profile of the leaf essential oil of *M. barteri*

The hydro-distillation of *M. barteri* leaves gave pale yellow oil (0.51%) with a floral scent. GC-MS qualitative and quantitative data on the component of Leaf essential oil of *M. barteri* are shown in Figure 1 and summarized in Table 1. Twenty-six (26) compounds were identified representing 91.5% of the oil constituent. Apocarotenoids were the main constituents identified in the leaf essential oil (49.4%) followed by non-terpene derivatives (38.5%). Minor quantifications were observed in oxygenated monoterpenes (2.7%) and monoterpene hydrocarbon (0.9%). The most abundant compound was found to be *(E)*-geranyl acetone (21.9%). Other apocarotenoids identified in leaf essential oil include (*E*)-α-ionone (8.9%), 3, 4-dehydro-β-ionone (5.4%), (*E*)-β-ionone (5.0%), (*E*)-β-damascenone (3.8%), β-cyclocitral (1.5%), β-cyclohomocitral (1.2%), Safranal (0.9%) and dehydro-ar-ionene (0.8%). Other minor constituents of the essential oil include oxygenated monoterpenes [*neo*-dihydrocarveol (1.8%) and geranial (0.9%)] and monoterpene hydrocarbon [dihydrocamphene (0.9%)]. Some non-terpenes and derivatives identified were hexanal (6.4%), 2-pentylfuran (5.3%), *(E, E)*-2,4-decadienal (3.6%), *(E)*-2-undecenal (3.0 %), (*E*)-2-hexenal (2.3%), 6-methyl-5-hepten-2-one (1.6%) and octanal (1.5%).

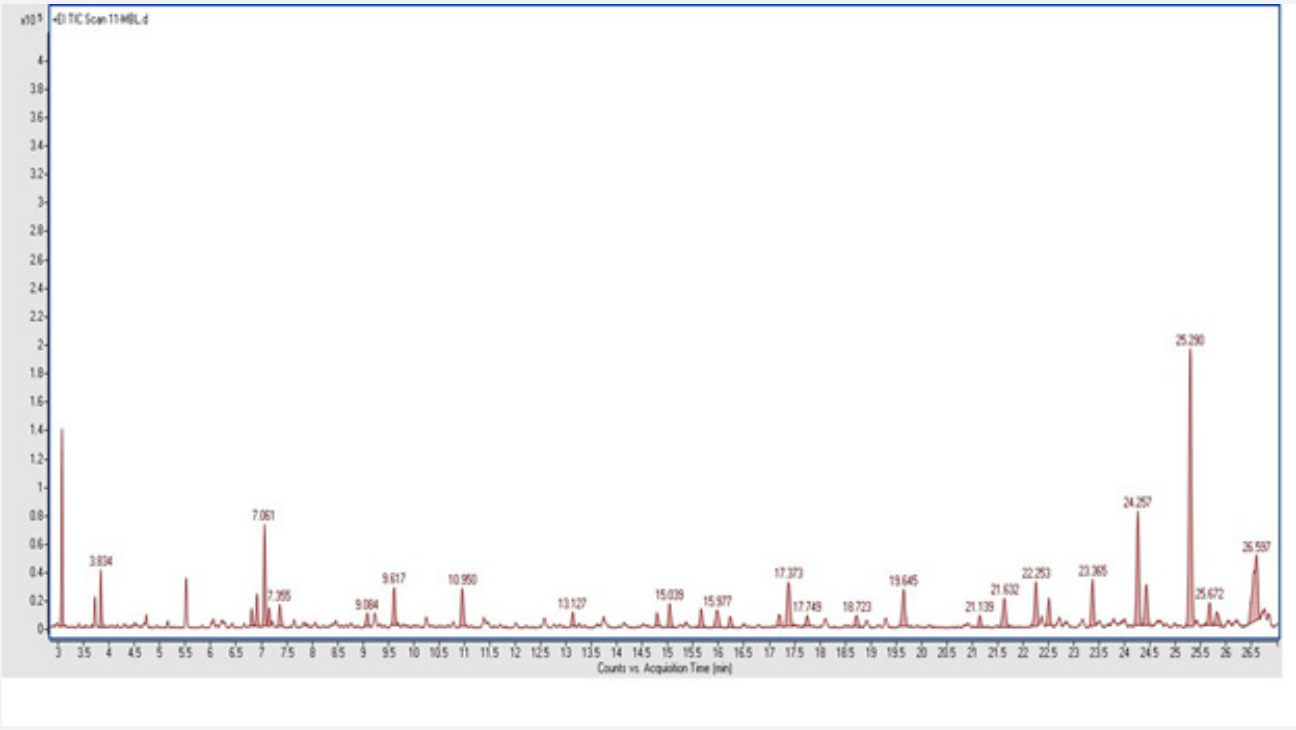

Figure 1: GC Chromatogram of the essential oil of M. barteri leaves.

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Table 1: Phytochemical Profile of leaves essential oil of M. barteri.

Serial Number	Identified Compounds	LRI	TIC (%)
1	Hexanal	802	6.4
2	(E)-2-hexenal	856	2.3
3	2,3-octanedione	984	0.8
4	6-methyl-5-hepten-2-one	987	1.6
5	2-pentylfuran	993	5.3
6	Octanal	1002	1.5
7	Dihydrocamphene	1053	0.9
8	2-methylbenzaldehyde	1068	2.6
9	Nonanal	1102	3.1
10	(E)-2-nonenal	1163	0.9
11	Safranal	1199	0.9
12	Decanal	1206	1.8
13	β-cyclocitral	1222	1.5
14	neo-dihydrocarveol	1228	1.8
15	β-cyclohomocitral	1256	1.2
16	(E)-2-decenal	1263	4.5
17	Geranial	1270	0.9
18	(E, Z)-2,4-decadienal	1293	1.1
19	(E, E)-2,4-decadienal	1316	3.6
20	dehydro-ar-ionene	1354	0.8
21	(E)-2-undecenal	1364	3
22	(E)-β-damascenone	1382	3.8
23	(E)-α-ionone	1427	8.9
24	(E)-geranyl acetone	1456	21.9
25	3,4-dehydro-β-ionone	1485	5.4
26	(E)-β-ionone	1488	5
Monoterpene hydrocarbons			0.9
Oxygenated monoterpenes			2.7
Apocarotenoids			49.4
Non-terpene derivatives			38.5
Total identified			91.5

LRI - Linear Retention Index, TIC: Total ion concentration in percentage

Cytotoxicity Assay

The nauplii were exposed to leaf essential oil of *M. barteri* at different concentrations for 24hrs. The number of mortalities were calculated to determine the effectiveness of the essential oil. The cytotoxicity of *M. barteri* essential oil and doxorubicin against nauplii (IC_{50}) was 2822.46 µg/mL and 114.92 µg/mL respectively.

Antimicrobial Activity of the leaf essential oil of *M. barteri*

Experimental data obtained from the *in vitro* antimicrobial activity of *M. barteri* leaf essential oil is summarized in Table 2. The essential oil of *M. barteri* leaves exhibited significant antimicrobial activities with inhibitory zones of 22-34 mm against bacterial strains and 16-25mm against fungal strains when compared to the controls (Gentamicin: 40-45 mm and Tioconazole: 33 mm).

Antioxidant Activity

Results of the scavenging ability of leaf essential oil of *M. barteri* on DPPH* assay and IC_{50} values are summarized in Table 3, Figure 2. The percentage inhibition of the essential oil was concentration dependent.

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Table 2: Inhibition Zones of leaf essential oil of M. barteri and Reference Standards.

Test Organisms	DMSO	Positive Control		Concentration (%)		
		Gentamicin	Tioconazole	100	50	25
Escherichia coli	(-)	42	N/A	34±0.00	29±0.00	26±0.00
Klebsiella pneumonia	(-)	45	N/A	33±0.00	28±0.00	27±0.00
Staphylococcus aureus	(-)	40	N/A	29±1.41	26±1.41	23±1.41
Bacillus subtilis	(-)	44	N/A	28±0.00	25±0.00	22±0.00
Candida albicans	(-)	N/A	33	25±0.00	22±0.00	20±0.00
Penicillium notatum	(-)	N/A	33	24±0.00	21±0.00	19±0.00
Aspergillus niger	(-)	N/A	33	23±1.41	19±1.41	16±1.41

The inhibitory zone was measured in millimeter, mm; N/A: Not applicable, (-): No inhibition, ± = Standard deviation.

Table 3: Antioxidant activity of leaves essential oil of M. barteri with percentage inhibition.

Test samples	% Inhibition at each concentration (µg/mL)				
	1000	500	250	125	62.5
MBL	90.04±0.0000	89.01±0.0000	88.95±0.0012	85.43±0.0000	84.73±0.0006
Ascorbic acid	93.05±0.0006	92.87±0.0006	92.78±0.0012	90.49±0.0001	90.40±0.0000
BHA	92.60±0.0006	91.96±0.0000	91.41±0.0000	91.32±0.0000	90.68±0.0012

MBL: leaf essential oil of M. barteri, BHA: Butyl hydroxyanisole, ± = Standard deviation.

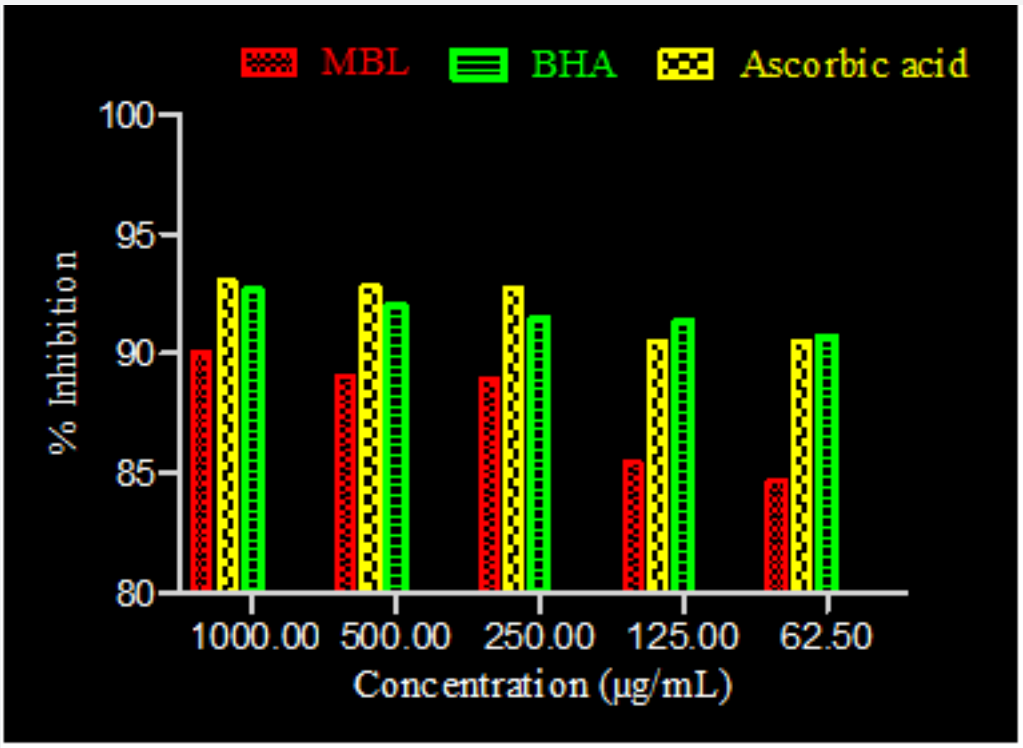

Figure 2: Percentage inhibition of leaf essential oil of M. barteri antioxidant activity compared with the reference standard.

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Discussion

The reputed medicinal properties of *M. barteri* leaves in folk medicine might be due to the presence of relatively high amounts of apocarotenoids in the essential oil. *(E)*-geranyl acetone has been reported to possess strong antimicrobial activity [20-22]. β-Ionone has been shown to demonstrate potent anticancer activity both *in vitro* and *in vivo* and also suppresses DMBA-initiated mammary cancer in the rat [23]. α-ionone and β-ionone inhibited growth against algae [24] and are used in cosmetics such as perfumes [25]. Apocarotenoids also have wide applications in food, feed, nutritional, pharmaceutical and personal care. Their remarkable nutrition and health importance cannot be overemphasized [26-28].

Brine shrimp lethality assay is a method employed to evaluate the level of toxicity in plant extract and others based on the ability of the plant extract to kill a laboratory cultured larvae (*nauplii*) [17]. According to standard guidelines for determining the level of toxicity of samples, $IC_{50} > 1000\ \mu gmL^{-1}$ is considered nontoxic [29]. Thus, the essential oil in this study is non-toxic (IC_{50} of 2822.46 µg/mL) when compared to the doxorubicin (IC_{50} of 114.92 µg/mL).

The antimicrobial activities of *M. barteri* leaves essential oil and the test organisms were concentration dependent. The essential oil showed significant antibacterial activity against both the gram-positive (23-28 mm) and gram-negative bacteria (26-34 mm). Generally, the gram-negative reference strains were more sensitive to *M. barteri* leaves essential oil than the gram-positive at all concentrations. Furthermore, *E. coli* and *B. subtilis* were more susceptible to the essential oil among the test strains by inhibitory zones of 26-33 mm and 22-28 mm respectively. With the antifungal assay, *M. barteri* leaves essential oil was most active against *Candida albicans* by a zone of inhibition of 20-25mm. Essential oils that was dominated with apocarotenoids such as *(E)*-geranyl acetone, α-ionone and β-ionone were reported to inhibit the growth of some microorganism [20-22,24]. The polar content such as apocarotenoids in the leaves essential oil may account for the strong inhibition of growth in the test microorganisms as polar compounds have been found to have better interaction with microorganisms - cell walls leading to their death [30].

DPPH* assay is considered a valid, accurate, easy and economic method to evaluate radical scavenging activity of antioxidants since the radical compound is stable [19, 31]. The mechanism of this assay follows the change in color of DPPH from purple to yellow as the radical is reduced by the antioxidant agent either by donating hydrogen atom or electron transformation of DPPH into its reduced form DPPH-H, becoming a stable diamagnetic molecule [30]. It was observed that the leaf essential oil of *M. barteri* exhibited significant scavenging ability at all concentrations with percentage inhibition between 90.04-84.73 (Figure 2 and Table 3), comparable to that of ascorbic acid (93.05-90.40) and BHA (92.60 - 90.68). Statistical analysis of the data subjected to analysis of variance (ANOVA) showed that there was significant difference between the leaf essential oil and the reference standard at $p<0.05$. Thus, the presence of apocarotenoids in the essential oil might be responsible for the antioxidant activity [32].

Conclusions

Twenty-six compounds were identified in the leaf essential oil of *M. barteri*. The essential oil is non-toxic and displayed significant antioxidant and antimicrobial activities. The essential oil is rich in apocarotenoid (49.4%). Apocarotenoid has been found to exhibit numerous biological activities in *in-vitro* and *in-vivo* experiments, including free radical scavenging, sunburn preventive, chemo preventive, antimicrobial, insect repellent and attractive effects. The essential oil of *M. barteri* displayed a significant free radical scavenging activity with percentage inhibition ranging from 90.04-84.73%. The leaf essential oil showed susceptibility against bacterial and fungal strains and the brine shrimp lethality test revealed that the essential oil was non-toxic. These results justify the medicinal applications of *M. barteri* in ethno-medicine.

Authors' Contributions

PAO, Organic Chemistry-Ph.D. was involved in all the laboratory work, conceptualization, result interpretation and writing (editing); MGI- M.Sc. Organic Chemistry-Ph.D. candidate carried out the sample collection, extraction of the essential oil, cytotoxicity, antioxidant, antimicrobial assays, results interpretation and write up; OOO-Ph.D. was involved in the GC-MS analysis and interpretation of the essential oil; GKO-Ph.D. was involved in the antioxidant assay; GF, Professor (Associate) of Pharmacy-Ph.D. carried out the GC-MS analysis of the essential oil. All authors read and approved the final manuscript.

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