GC-MS FINGERPRINT OF *Boswellia dalzielli* HUTCH (FAMILY: BURSERACEAE) AND ITS BIOACTIVITY AGAINST *Plasmodium falciparum*

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Abstract: The antiplasmodial activity of *Boswellia dalzielli* (Burseraceae) Hutch leaves 70% aqueous methanol crude extract and its C-18 resin flash chromatography fractions were evaluated. The study was carried out using the SYBRGreen 1dye assay in an *in vitro* culture of parasitized red blood cells. Microscopy was implored to serve for validation of the results obtained by the SYBRGreen dye 1 assay. The leaf crude extract showed a poor bioactivity with an IC_{50} of 47 µg/ml against the 3D7 strain of *Plasmodium falciparum.* However, upon fractionation, one of the fractions gave an IC_{50} of 17.8 µg/ml activity but with a correspondingly high level of haemolytic effect. The leaves of *Boswellia dalzielli* showed no cytotoxicity against the liver carcinoma cell line (HUH-7) but it was highly lytic against the Red blood Cells. This attributes shows it's none selectivity in its bioactivity against the *Plasmodium* parasites. Thus, having a very low therapeutic index and as such not a promising drug candidate against malaria, except if used in synergy with other compounds that could buttress its antiplasmodial activity and attenuate its haemolytic effect. Its GC-MS fingerprint displays an array of rich phytocompounds that could be useful to man and other organisms for the improvement of life conditions.

Keywords: Antimalarials, *Boswellia dalzielli*, SYBRGreen 1 assay, *Plasmodium falciparum* 3D7 strain

Introduction

Plasmodium falciparum causes severe complications in humans; inclusive of which is, its ability to cause cerebral malaria, severe anaemia, acute renal failure, hypoglycemia and pulmonary infection. The two features that actually separate *P. falciparum* from the other human malaria are its ability to attack erythrocytes of all ages, causing high parasitaemia, and the capability to adhere to vascular endothelium through sequestration (Okwa, 2003). Malaria accounts for 6.5% of abortions, 15% of premature deliveries and 0.7% death in foetus (WHO, 2010). Malarial infection is still a public health burden in the tropics and there is urgent need for more interventions in terms of therapeutics and vaccine formulations.

Boswellia dalzielii (Burseraceae) is a tree plant, abundantly found in north-western Nigeria, where the Hausa speaking people refer to it as Hano or Harrabi. This plant is very popular among the locals as a potent source of ethnomedicine. The extract from its leaves is used for the treatment of diarrhea in poultry and the stem bark extract has been reported to help in hepato-strenghtening.

In this study, we explored the antiplasmodial potency of the leaves extract of *Boswelia dalzielli* with the aim of identifying lead antimalarial compounds for possible future development of new antimalarial drugs.

Materials and Methods

Plant materials

The leaves of *Boswelia dalzielli* (W16L) were collected from the wild in Zaria, northern Nigeria (11.0667° N, 7.7000° E), West Africa. They were properly identified and documented with the voucher number 900121 at the Herbarium Section of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria. The plant material was coded using number and alphabet that indicate the part of the plant in use in this study.

Plant extract preparation

The plant leaves were air dried at room temperature for 2 weeks, after which they were pulverized. 5 g of the powdered dried leaves were macerated in 5.5 Litres of 70% (v/v) aqueous methanol and kept in a shaker (at 130 rpm, 28ºC) for 17 h. Filtrate was poured into a Rotary evaporator for condensation and dryness (Rotavapor Buchi R210/V, Vacuum controller V-850) at a vacuum pressure of 109 mbar, temperature of 50ºC and at a speed of 4 rpm. After which the crude extract obtained was kept safe in a refrigerator till usage. An extract yield of 5.6% was obtained.

Bio-assay guided fractionation of test materials using the C-18 resin flash column

Five (5) grams of the test materials were dissolved in 20 ml of milliQ water to which 5 ml of 100% methanol was added to dissolve the pellets. 10 g of C-18 resin was loaded onto the sintered glass column and washed down with 20 ml of 100% methanol and 50 ml of water separately. Samples were loaded on column and 100 ml of 5% aqueous methanol were used to elute them as flow through or unbound materials. Step down gradient elution of sample materials was done using varying percentages of aqueous methanol of 200 ml volume each as follows: 0, 10, 20, 40, 60, 80 and 100% methanol, respectively (Kaushik *et al*., 2013). The resultant eluents were dried and tested *in vitro* for their biological activities against *Plasmodium falciparum*.

Dilutions of test materials and control drugs

Parent stocks of the test materials were formulated by dissolving the test materials at 25 mg/ml in Dimethyl Sulphoxide (DMSO). 10 µl of these parent stock solutions were aliquoted with mixing into different wells of the micro titer plate each of which contained 90 µl of distilled water. The resulting working dilutions were now 2.5 mg/ml in 10% DMSO. The working dilutions (50 µl) were then two fold diluted using 8 channel multipipettes in triplicate wells containing 50 µl of 10% DMSO to obtain a series of concentrations all in 10% DMSO. This plate containing working dilutions in 10% DMSO was designated as master

plate. 4 µl of each concentration in triplicate from the master plate were then transferred to three wells of the daughter plate Triplet wells containing 4 µl chloroquine (1 mM) and triplet wells with 4 µl c RPMI served as positive (100% growth inhibition) and negative (full growth) controls. Finally a multipipette was used to dispense 96 µl of ring stage synchronized culture of malaria parasite to give 1% parasitemia and 2% hematocrit. In the process, the samples undergo 25 fold dilution of solute to a final DMSO concentration of 0.4% (v/v) in cRPMI (this concentration of DMSO is non-toxic to the growth of malaria parasite). The cultures were then incubated at 37º C under standard gas composition (5% O_2, 5% CO_2, 90% N_2).

Plasmodium falciparum in vitro cultivation and the antiplasmodial activity assay

The crude leaves extract of *Boswelia dalzielli* plant and its C-18 resin flash chromatography fractions were subjected to an *in vitro* antiplasmodial screening using the chloroquine sensitive *Plasmodium falciparum* 3D7 (*Pf* 3D7). This was maintained in media composed of RPMI 1640 containing 0.2% sodium bicarbonate, 0.5% albumax I, 45 µg/mL hypoxanthine, 50 µg/mL gentamicin and incubated under a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 in a CO_2 incubator at 37ºC.

SYBR green I-based fluorescence assay was used as described (Smilkstein *et al*., 2004). Sorbitol synchronized ring stage parasites were incubated under normal culture conditions at 2% hematocrit and 1% parasitemia in varying concentrations of the test samples. Chloroquine (1µM) was used as positive control (zero growth) while, DMSO at 0.4% (v/v) was used as negative control (100% growth). After 48 hours of incubation, 100 µl of SYBR Green I solution [0.2 µl of 10,000 X SYBR Green I (Invitrogen)/mL] in Lysis Buffer [Tris (20 mM; pH 7.5), EDTA (5 mM), Saponin (0.008%; w/v), and Triton X-100 (0.08%; v/v)] was added to each well and mixed twice gently with multi-channel pipette and incubated in the dark at 37°C for 1 h. Fluorescence was measured with a Victor fluorescence multi-well plate reader (Perkin Elmer) with excitation and emission wavelength bands centered at 485 and 530 nm, respectively. The fluorescence counts were plotted against the drug concentrations and the 50% inhibitory concentration (IC_{50}) was determined by analysis of dose-response curves (Kaushik *et al*., 2013; Amlabu *et al*., 2017).

MTT assay to test for cytotoxicity against HUH-7 cell line

The liver carcinoma cell line (HUH-7 cell line) was cultured in RPMI containing 10% fetal bovine serum, 0.21% sodium bicarbonate (Sigma) and 50 µg/ml gentamycin (complete medium). Cells (10^4 cells/200µl/well) were seeded into 96 well flat-bottomed tissue culture plates in complete medium and drugs or test materials were added after 24 h of seeding and then left to incubate for 48 hrs at 37°C in a 5% CO_2 incubator. Thereafter, 10% DMSO was added in triplicate wells at the beginning of the assay to give 0% growth (Negative control) while three other wells were left without treatment to give a 100% growth (Positive control). MTT stock solution (20 µl) at a concentration of 5 mg/ml in phosphate buffered saline was added to each well, mixed gently and incubated for another 4 h. The plates were then spun for 5 min at 1,500 rpm and the supernatants removed. Then 100 µl of DMSO was added as a stop agent, following which the formation of Formazon was read on a microtiter plate reader (Versa max tunable multi-well plate reader) at 570 nm wavelength. Analysis of dose-response curves was used to determine the 50% cytotoxic concentration (TC_{50}) of test samples. The selectivity index/ therapeutic index was calculated as the ratio of TC_{50} HUH-7/ IC_{50} *Pf*3D7 (Mossman, 1983).

Haemolysis assay against human red blood cells

The Human Red Blood cells were subjected to a haemolysis assay to check the concentration dependent haemolytic effect of the test materials (W16L) as described by Kazi *et al*. (1994). At the end of the assay the supernatant was read on a microtiter plate reader (Versa max tunable multiwell plate reader) at 415 nm. The 50% haemolytic concentration (HC_{50}) of the test materials was determined by analysis of dose-response curves.

Morphological changes of Plasmodium due to effects of test materials

A study of the effect of the test samples on the morphology of the parasites was conducted using microscopy after incubation of parasites with the test materials. The test samples were incubated with (synchronized ring stage parasites) for 48 hrs, thin smears of the incubated cells on glass slides were made and stained with Giemsa stain. The slides were viewed under a microscope (Nikon Eclipse 50i) to observe the nature of the parasite cells after treatment with the test samples. The reference drugs and the untreated parasites served as positive and negative controls, respectively.

GC-MS analysis of the fractionated test materials for compounds identification

The identification of the chemical composition of the crude leaf extract was carried out using the machines with the following specifications: Shimadzu QP2010 with a DB-5 column (30 m, film 0.25 mm, internal diameter 0.25 mm). Temperature of the column was programmed from 45 to 270ºC at 5ºC/min, the injector and detector temperature for the analysis was 250ºC. Helium was used as the carrier gas at a flow rate of 1.5 ml/min. The mass spectrometer was operated in electron impact ionization mode at 70 eV. The chemical constituents of the plant leaf extract and their structures were identified after correlation of their recorded mass spectra with those obtained from the WILEY8.LIB and NIST08.LIB library spectra provided by the software of the GC-MS system (Rosa *et al*., 2003). The compounds were identified based on their Retention time (RT), Molecular formula (MF), Molecular weight (MW). This study was carried out in order to determine the classes of compounds present in this plant species; which could serve as a taxonomic fingerprint with reference to its type locality, its method of extraction and also provide information on its natural constituents for future reference and usage.

Results and Discussion

In this study, the antiplasmodial activity of the aqueous methanol crude leaf extract of *Boswellia dalzielii* was tested against the chloroquine sensitive strain (3D7) of *Plasmodium falciparum*. The results gotten are itemized in Table 1, Figs. 1, 2 and 3. The antiplasmodial activities obtained from the crude extract and the C-18 resin flash column fractioned materials were not promising as potential drug candidates. The bioassay showed that the plant has slight or weak antiplasmodial activity and it is highly haemolytic. The fraction with the best antiplasmodial activity showed a corresponding high haemolytic effect. However, upon fractionation, the fraction without haemolytic effect showed no antiplasmodial activity and no cytotoxicity against the Human liver carcinoma cell line (HUH-7).

Table 1: The antiplasmodial, cytotoxicity and haemolytic profile of *Boswellia dalzielli* (W16L) crude leaf extract and its C-18 resin fractions

S/N	W16L fractions	HC_{50} µg/ml RBC	IC_{50} µg/ml (*Pf* 3D7)	TC_{50} µg/ml HUH-7	Therapeutic Index (TC_{50}/ IC_{50})
1	FT	32	>50	-	-
2	0%	60	>50	-	-
3	10%	61	>50	-	-
4	20%	63	>50	-	-
5	40%	>100	>50	>100	2
6	60%	16	27	>100	3.7
7	80%	38	17.8	-	-
8	100%	52	-	-	-
9	Crude Extract	<1.56	47	-	0.03
10	Untreated RBCs % Haemolysis =0.3				

The biological activity of this plant extract against the plasmodial cells is not clear if the plant actually kills the *Plasmodium* parasites selectively or the parasites gets to die due to the plant's highly lytic activity against the red blood cells which houses the *Plasmodium* parasites, and thus upon the lysis of the Red Blood Cells, then the parasites get to die invariably. Also, it was observed that the fraction with the highest haemolytic effect showed no cytotoxicity against the Human liver carcinoma cell line which means that the haemolytic agents in the plant material do not confer any harmful effect on the mammalian liver cells. The non-cytotoxic effect of *B. dalzielii* extract on the human liver carcinoma cells observed in this study, agrees with the reports of Aliyu *et al*. (2007) who reported the hepato-strengthening activity of *Boswellia dalzielii* aqueous bark extract on rat liver function. However, there are no reports on the antiplasmodial activity of this plant so far, but Atawodi *et al*. (2011), reported the antitrypanosomal activity both *in vitro* and *in vivo* of the methanolic leaf extract of *Boswellia dalzielii*. Also, the antispasmodic activity of the methanolic stem bark extract of *Boswellia dalzielii* was reported by Hassan *et al*. (2009) which justify its use traditionally in alleviating gastrointestinal disorders. Several works have been carried out on the antimicrobial activity of the extracts of *B. dalzielii* and no activity has been obtained on the aqueous stem bark crude extracts (Nwinyi *et al*., 2004), rather antibacterial activity was reported on the ethanolic stem bark crude extract (Olukemi *et al*., 2005).

The results of the GC-MS fingerprinting of the crude extract of *Boswellia dalzielii* in this study, revealed the presence of compounds such as tau.-Cadinol, n-Hexadecanoic acid, tetrahydroabietic acid, dronabinol, fumaric acid, ethyl 2-methylallyl ester etc (Table 2); which have been reported to be in use as antioxidants and also as therapeutics for the treatment of various ailments such as psoriasis, necrobiosis lipoidica, granuloma annulare, sarcoidosis, gliomas, CNS neoplasms and other solid tumours, amongst others ailments.

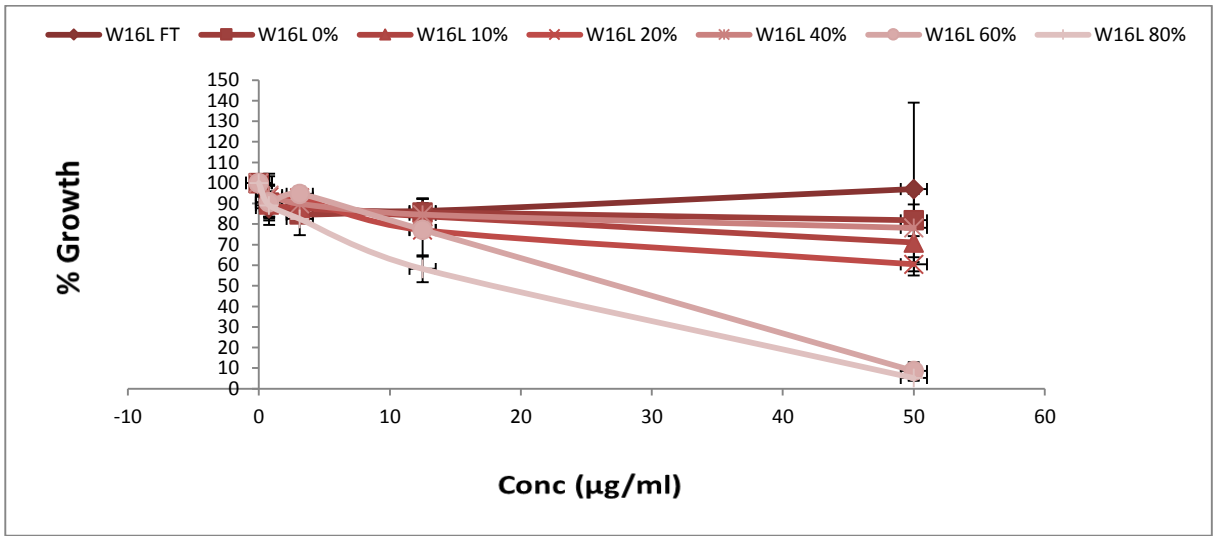

Fig. 1: The antiplasmodial activity profile of *Boswellia dalzielli* (W16L) C-18 resin flash chromatography fractions. Showing poor antiplasmodial activity

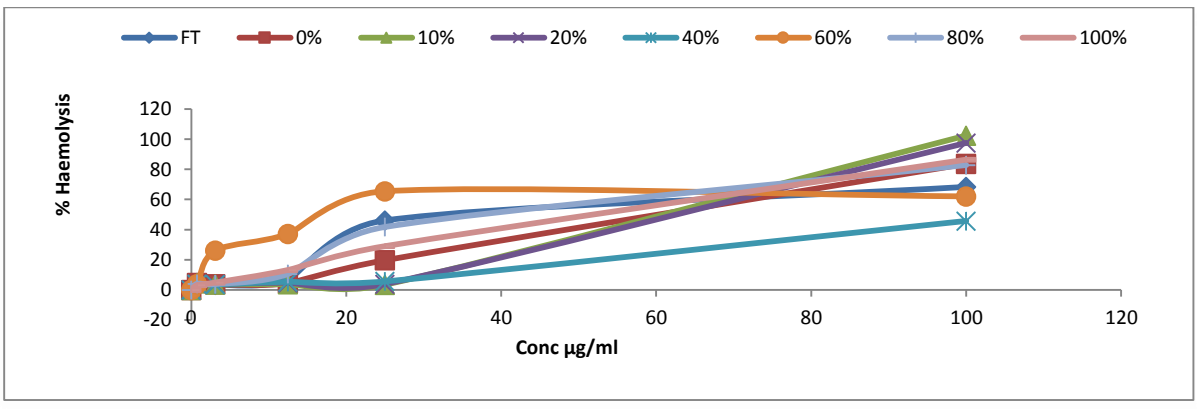

Fig. 2: Haemolysis assay profile of *Boswellia dalzielli* (W16L) C-18 resin flash chromatography fractions against the human red blood cells. Showing haemolysis except at 40% C-18 fraction

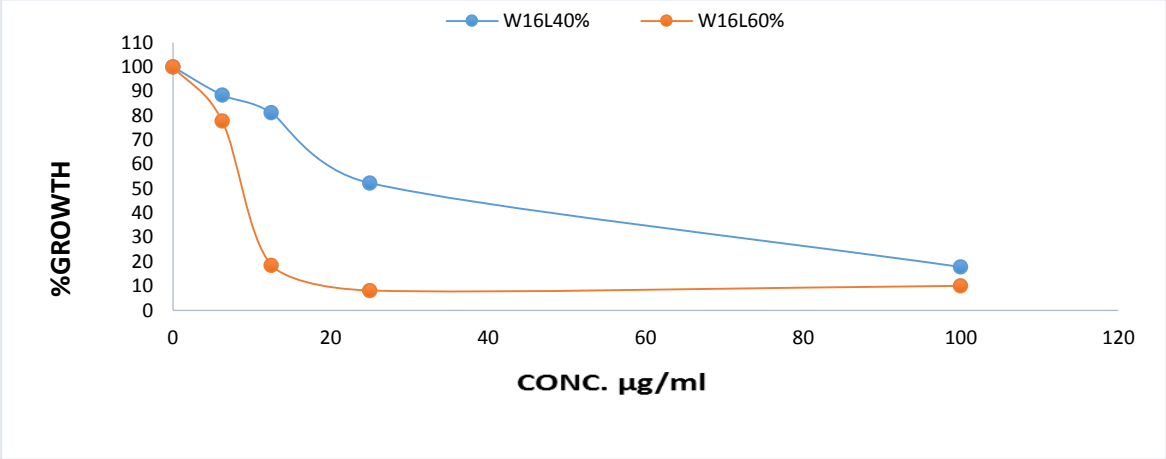

Fig. 3: Cytotoxicity profile of some *Boswellia dalzielli* (W16L) C-18 flash chromatography fractions against HUH-7 mammalian cell lines. Showing no cytotoxicity against the mammalian cell line

Fig. 4: Picture of *Boswellia dalzielli* plant

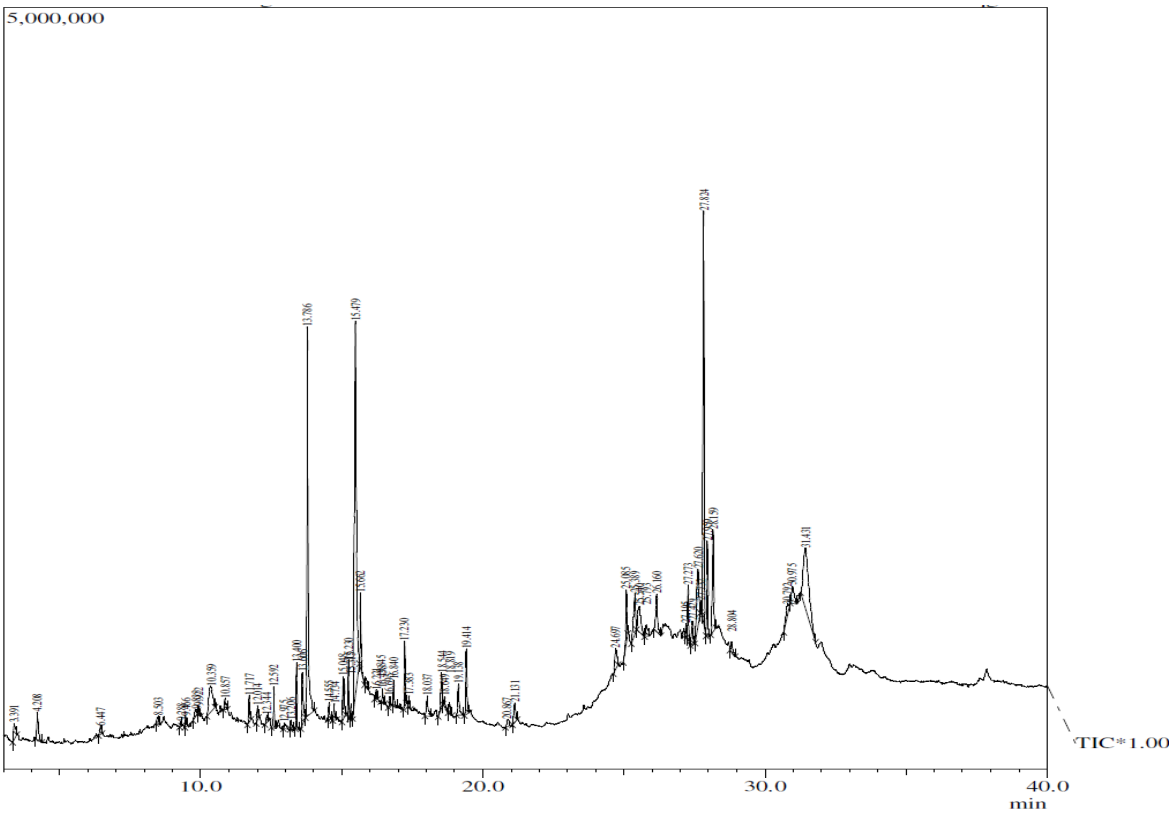

Fig. 5: Gas chromatography –mass spectrometry (GC-MS) chromatograph of 70% aqueous methanol crude leaves extract of *Boswellia dalzielli* (W16L) plant.

Table 2: Phyto-components identified in *Boswellia dalzielli* (W16L) 70% aqueous methanol crude leaves extract by GC-MS

Peak No.	RT	Name of Compound	MF	MW	Peak Area%
1	3.391	1-(6-oxabicyclo[3.1.0]hex-1-yl)ethanone	$C_7H_{10}O_2$	126	0.77
2	4.208	4h-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	1.04 -
3	6.447	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0.29
4	8.503	–	–	–	0.35
5	9.288	2(4h)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (r)-	$C_{11}H_{16}O_2$	180	0.12
6	9.466	Dodecanoic acid	$C_{12}H_{24}O_2$	200	0.27
7	9.833	.Alpha.-d-galactopyranoside, methyl	$C_7H_{14}O_6$	194	0.39
8	9.922	Ethanone, 1-(4-hydroxy-3-methoxyphenyl)-	$C_9H_{10}O_3$	166	0.19
9	10.359	1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid	$C_7H_{12}O_6$	192	2.32
10	10.857	.Alpha.-d-glucopyranoside, 1-o-methyl-4-hexyl-	$C_{13}H_{26}O_6$	278	0.38
11	11.717	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.74
12	12.014	2(4h)-Benzofuranone, 5,6,7,7a-tetrahydro-6-hydroxy-4,4,7a-13 12.344 166239 0.25 e-8-methyl-7-dodecen-1-ol acetate	$C_{11}H_{16}O_3$	196	0.39
14	12.592	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268	0.93
15	2.975	–	–	–	0.22
16	13.206	(9e,12e)-9,12-Octadecadienoyl chloride	$C_{18}H_{31}ClO$	298	0.30
17	13.400	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	1.27
18	13.606	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254	1.83
19	13.786	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	11.04
20	14.555	9-Octadecenoic acid (z)-	$C_{18}H_{34}O_2$	282	0.44
21	14.734	Heptadecanoic acid	$C_{17}H_{34}O_2$	270	0.47
22	15.048	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	1.44
23	15.230	Phytol	$C_{20}H_{40}O$	296	1.13
24	15.315	Octadecanoic acid, methyl ester	$C_{19}H_{38}O2$	298	0.27
25	15.479	Oleic acid	$C_{18}H_{34}O_2$	282	18.15
26	15.662	Octadecanoic acid	$C_{18}H_{36}O_2$	284	1.62
27	15.845	3,6-Dimethyl-1-heptyn-3-ol	$C_9H_{16}O$	140	0.35
28	16.221	–	–	–	0.22
30	16.695	2-[12-(2-Oxiranyl)dodecyl]oxirane	$C_{16}H_{30}O_2$	254	0.27
31	16.840	Hexadecanoic acid, 1-[[[(2-aminoethoxy)hydroxyphosphinyl]	$C_{37}H_{74}NO_8P$	691	0.58
32	17.230	Cyclohexane, eicosyl-	$C_{26}H_{52}$	364	1.41
33	17.383	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	324	0.20
34	18.037	Oleoyl chloride	$C_{18}H_{33}ClO$	300	0.89
35	18.544	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (e,e,e)-	$C_{57}H_{104}O_6$	884	1.20
36	18.649	Palmitoyl chloride	$C_{16}H_{31}ClO$	274	0.32
37	18.819	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	568	0.36
38	19.138	Octadecanal	$C_{18}H_{36}O$	268	1.13
39	19.414	Cyclohexane, eicosyl-	$C_{26}H_{52}$	364	1.93
40	20.867	16-Trimethylsilyloxy-9-octadecenoic acid, methyl ester	$C_{22}H_{44}O_3Si$	384	0.35
42	24.697	1,3-Cyclohexadecanedione, 6-nitro-	$C_{16}H_{27}NO_4$	297	1.09
43	25.085	Dehydroergosterol 3,5-dinitrobenzoate	$C_{35}H_{44}N_2O_6$	588	1.56
44	25.389	–	–	–	2.08
45	25.540	Trilinolein	$C_{57}H_{98}O_6$	878	1.98
46	25.793	Hexadecanal, 2-methyl-	$C_{17}H_{34}O$	254	0.59
47	26.160	7,7-Dimethyl-10-phenyl-5-m-tolyl-6,7,8,10-tetrahydro-5h-indeno	$C_{31}H_{27}NO_2$	445	1.76
48	27.195	2-Anthracenecarboxylic acid, 5,8-dihydro-6,9-dihydroxy	$C_{23}H_{26}O_6$	398	0.26
49	27.273	27-Norergosta-5,25-dien-3-ol, 25-ethyl-, (3.beta.,24.xi.)-	$C_{29}H_{48}O$	412	1.33
50	27.429	-			0.92
51	27.620	Benzo[3,4]cycloocta[1,2-f][1,3]benzodioxol-8-ol, 5,6,7,8-tetrahydro	$C_{23}H_{26}O_6$	398	3.19
52	27.732	-	-	-	0.20
53	27.824	7-(Trimethylsilyloxy)-3-[4-(trimethylsiloxy)phenyl]-4h-1	$C_{21}H_{26}O_4Si_2$	398	12.01
54	27.959	Dibenzo[4,5:6,7]cycloocta[1,2-c]furan-1(3h)-one, 3a,4,13,13a-	$C_{23}H_{26}O_7$	414	2.61
55	28.159	Dibenzo[a,c]cyclooctene-5-ol, 5,6,7,8-tetrahydro-1,2,3,9,11-pentamethoxy-	$C_{25}H_{32}O_7$	444	3.21
56	28.804	2,4-Dibenzyl-5,8-dimethoxy-6-methyl-1-naphthol	$C_{27}H_{26}O_3$	398	0.45
57	30.792	Undecanal, 2-methyl-	$C_{12}H_{24}O$	184	0.72
58	30.975	10-Undecen-1-al, 2-methyl-	$C_{12}H_{22}O$	182	1.11
59	31.431	2-Hydroxy-3-(palmitoyloxy)propyl (9e)-9-octadecenoate	$C_{37}H_{70}O_5$	594	7.88

Conclusion

The poor antimalarial potential of *Boswellia dalzielii* leaves extract is reported in this study using SYBRGreen 1 dye assay and validated with microscopic study. This report shades more light on the other usage of this plant leaves other than those previously reported but not as an antimalarial remedy. It is recommended that other works be carried out on this plant to test its bioactivity against other pathogens of medical importance in order to present it as possible source of some natural products due to its array of compounds as revealed by the GC-MS fingerprint.

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