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Volatile Constituents and Toxicity Profile of the Leaves, Stem Bark and Root Bark Essential Oils of Holarrhena Floribunda and Crescentia Cujete

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Abstract: Essential oils are highly concentrated substances extracted from flowers, leaves, stems, roots, seeds, barks, resins, or fruit rinds. The increased interest in creating a compendium of plant essential oils for the purpose of discovering drugs from natural source led to the characterization of the leaves, stem bark and root bark of *Holarrhena floribunda* and *Crescentia cujete*. The essential oils from the plants parts were extracted by means of hydrodistillation using an all glass Clevenger apparatus while the chemical constituents were determined by Gas Chromatography-Mass Spectroscopy technique. The toxicity of the essential oils was tested using Brine shrimps (*Artemia salina*). The GC-MS results identified 5, 8, and 7 components in *H. floribunda* (leaves, stem bark and stem bark respectively) while leaves, stem bark and root bark of *C. cujete* had 15, 11 and 10 constituents respectively. The compounds found in high quantity in the essential oils of *H. floribunda* are friedelan-3-one (22.85%), sesquirosefuran (31.93%), octadec-9-enoic acid (46.28%), and longifolene (59.77%) while the major components in the oils of *C. cujete* are cyclotetradecane (13.75%), diisooctylphthalate (33.96%), and phytol (46.33%). The LC_{50} value ranged from 10.85 to 288.76 (µg/mL) which was a pre-test for toxicity potential. The essential oils of *C. cujete* stem bark, *C. cujete* root bark and *H. floribunda* root bark were the most toxic with LC_{50} 10.85, 16.54 and 36.33 µg/mL respectively. The level of toxicity of these essential oils is an indication of the pharmacological properties the plants may possess.

Keywords: Essential oil, *Crescentia cujete*, *Holarrhena floribunda*, Longifolene, Toxicity

INTRODUCTION

Holarrhena floribunda, also known in English as False rubber tree, belongs to the family Apocynaceae (Letouzy, 1972). It is known as *ire-oju-ona* in Yoruba, *gaman sauwa* in Hausa and *mba* in Igbo (Nigeria). It is a tree or shrub about 17 m high. It is widely distributed in the centre and west regions of Cameroon. The stem bark is used in Cameroon to treat various ailments such as abdominal pains, nausea, indigestion and diarrhea (Berhaut 1971). The stem bark of *H. floribunda* is febrifuge and could be a quinine substitute, since it showed remarkable inhibitory activity against drug-resistant strains of *Plasmodium falciparum* (Fortie et al., 2006). This plant has been found to display a wide spectrum of biological and pharmacological activities such as antibacterial (Bogue et al., 2007), analgesic (Udobre et al., 2014), hypoglycemic (Gnangoran (2012) and antiamiboedal (Goutarel (2012).

The common calabash tree (*Crescentia cujete*) is of the Curcubitaceae family and has been cultivated widely throughout the new world tropics since prehistoric times; its exact native range is uncertain. It is a small tree with light green bell-shaped flowers (5 to 6.5 cm long) that are borne singly on stout stalks on the trunk and branches. The flowers are bat-pollinated and are produced irregularly throughout the year. The large fruit has a thin hard shell and whitish pulp and does not split open. The many seeds are dark brown, thin, and flat. Like some other fruits of species in the family Bignoniaceae, the fruits of this species possess nectar-producing nectaries. In some other species in this family, these nectaries have been shown to attract ants that drive away animals that feed on the plants (Elias, 1978). Ejelonu *et al*. 2011 investigated the proximate composition of *C. cujete* and found out that the value of the fat, protein, nitrogen, crude fibre, moisture content, sucrose, fructose, galactose and energy content were quite high. Nandita *et al*. 2014 also investigated the antioxidant properties of the ethanol extracts and fractions of leaves and stem bark and reported the stem bark as having the highest antioxidant activity.

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This paper explores the characterisation of the chemical constituents and toxicity profile of the essential oils of *H. floribunda* and *C. cujete* in continuation of our studies to create a compendium of Nigerian plant essential oils.

Experimental

Plant collection and preparation.

The leaves, stem bark and root bark of *H. Floribunda* and *C. Cujete* were collected in March, 2016 from the Botanical Garden, University of Ibadan, Ibadan, Nigeria. The samples were identified at the Herbarium, Botany department, University of Ibadan, Ibadan by Mr. Donatus.

The fresh samples were air-dried and pulverized then stored in air-tight polythene bags prior to extraction.

Isolation of essential oils

Hydro-distillation was carried out using an all-glass Clevenger apparatus for the extraction of the essential oils. Exactly 200 g of each of the pulverized samples was weighed and carefully placed in a 5 L round bottom flask and water was added until the sample was fully immersed. The extraction process was then carried out for each of the weighed sample for 3 hours according to the British pharmacopoeia specification (1980). The sample vial was then stored in the refrigerator at 4 ^{o}C until further analysis.

Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis.

The essential oils were analysed using GC-MS Agilent 7890A gas Chromatograph coupled with MS Agilent Technologies 5975 series MSD. The capillary column type was an HP-5ms with column length 30 m; internal diameter 0.25 mm and 0.25 µm film thickness. The carrier gas was helium at constant flow rate of 1 mL/min. The initial column temperature was set at 60 ℃ for 2 mins and was then increased at the rate of 10 ℃/min to 240℃ for 6mins.

Identification of the constituents

The constituents of the essential oils were identified by comparing their retention times with an analysis done under the same temperature-programmed conditions for n-alkanes and the oil under the same chromatographic conditions. Individual compounds were identified by comparing their mass spectra with the internal reference mass spectra library or with authentic compounds. Confirmation of identity was done by comparing their retention times with the GC-MS and mass spectra from literature data (Adams, 2007; Joulain and Koenig, 1998).

Brine shrimp assay

Sea water was collected from the ocean in Lagos State, South West, Nigeria. The shrimps (*Artemia salina*) were purchased from Felimar Aquaculture Centre, Ijebu-ode, Ogun State (produced by Coppens International, Helmond, Holland). Sea water (200 mL) was put in a tank or hatching chamber and shrimp eggs added. The tank or hatching chamber was a plastic bowl, partitioned in to two compartments. The partition was perforated such that the nauplii could swim through to the other side after hatching. The eggs were allowed to hatch for 48 h and mature as nauplii at room temperature. The nauplii were then harvested with a pipette after attracting the organism to one side of the vessel with a touch-light source.

The essential oils were prepared in sea water into vials at 1000, 100, and 10 µg/mL (each test in triplicates). The essential oils had been previously dissolved in 2mL of Dimethylsulfoxide (DMSO) since they are not soluble in water and 0.5 mL each of the dose level were introduced in a test-tube to which 4 mL of sea water added. Exactly 10 shrimps per test tube were added for each concentration and made up to 5 mL seawater to make 1000-10 µg/mL of final concentration of extract. After 24 h, the number of deaths over the number of total shrimps (survivors) was counted and recorded. The concentration killing fifty percent of the larvae (LC_{50}) was determined.

RESULTS AND DISCUSSION

The percentage yields of the essential oils are presented in table 1. The yields were calculated based on volume of essential oil to weight of dry plant sample (v/w).

Table 1: Percentage yields of the essential oils.

S/N	Plant Sample	Mass of Sample (g)	Yield of Oil (v/w) (%)
1.	HFL	200	0.55
2.	HFS	200	0.39
3.	HFR	200	0.63
4.	CCL	200	0.63
5.	CCS	200	0.71
6.	CCR	200	0.45

HFL = *Holarrhena floribunda* leaf, **HFS** = *Holarrhena floribunda* Stem bark
HFR = *Holarrhena floribunda* root bark
CCL = *Crescentia cujete* Leaf, **CCS** = *Crescentia cujete* Stem bark
CCR = *Crescentia cujete* root bark

Table 2: Chemical Composition of the essential oils of *Holarrhena floribunda* and *Crescentia cujete.*

S/N	RT	CONSTITUENTS	% COMPOSITION					
			HFL	HFS	HFR	CCL	CCS	CCR
1.	11.15	α-ionone	-	-	-	**5.74**	-	-
2.	12.39	(E)-β-ionone	-	-	-	**9.41**	-	-
3.	14.63	Farnesene	-	-	-	-	-	1.48
4.	15.61	τ-muurolol	-	**11.73**	-	-	-	-
5.	15.93	Longifolene	-	**59.77**	-	-	-	-
6.	15.94	(1S,4aS,7R,8aS)-1, 4a-dimethyl-7-(prop-1-en-2-yl) decahydronaphthalen1-ol	-	-	17.96	-	-	-
7.	16.67	2,6,10-trimethyltridecane	-	3.57	-	-	-	-
8.	18.43	Hentriacontane	-	-	-	-	4.74	-
9.	18.56	Phytane	-	**3.40**	-	**2.30**	-	**9.31**
10.	18.58	6-methyl-tridecane	-	-	-	-	11.46	-
11.	19.29	Hexahydrofarnesyl acetone	-	-	-	**5.62**	-	-
12.	20.73	7,9-di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	-	-	-	3.84	-	-
13.	20.76	Hexadecanoic acid	-	-	15.86	-	-	-
14.	20.87	Dodecanoic acid	-	-	0.96	-	-	-
15.	21.09	Isophytol	-	-	-	1.88	-	-
16.	22.63	2,4,4,6,6,8,8, heptamethyl -1-nonene	-	-	-	2.00	9.36	9.92
17.	22.64	Friedelan-3-one	-	-	**22.85**	-	-	-
18.	22.95	Heptadecanolide	11.29	-	-	-	-	-
19.	23.41	Lupeol	-	-	-	1.64	-	-
20.	23.59	Sesquirosefuran	-	-	**31.93**	-	-	-
21.	23.79	Phytol	-	-	-	**46.33**	-	-
22.	24.01	4-methyl octadecane	-	-	-	5.27	-	-
23.	24.25	Cyclotetradecane	-	-	-	3.76	-	-
24.	24.64	(Z)-p-menth-8(10)-ene-9-ol	-	-	-	-	2.25	-
25.	24.65	1-iodo-dotriacontane	-	-	-	1.92	-	-
26.	26.51	Eicosane	-	-	-	-	-	10.61
27.	26.52	(E)-9-Octadecenoic acid	-	-	-	-	4.51	-
28.	27.15	Octadecene	-	-	-	-	3.28	-
29.	27.19	3-methyl heptadecane	-	-	-	1.08	-	-
30.	27.70	1,2-benzisothiazole, 3-(hexahydro-1H-azepin -1-yl)1,1-dioxide	-	-	5.44	-	-	-
31.	27.71	(E) -9- eicosene	-	-	-	3.02	-	-
32.	27.72	(E)-15-heptadecenal	-	-	-	-	12.77	-
33.	27.73	10-methylundec-2-en-4-olide	-	-	-	-	5.97	-
34.	27.74	1-bromo-11-iodoundecane	-	2.85	-	-	-	-
35.	27.75	2-nitrofuran	-	-	-	-	-	7.43
36.	27.98	1-(ethenyloxy)-octadecane	8.44	-	-	-	-	-
37.	28.02	n-propyl-11-octadecenoate	17.57	-	-	-	-	-
38.	28.03	2-heptenoic acid, hexadecylester	-	5.02	-	-	-	-
39.	28.08	5-cyclohexadecen-1-one	12.23	-	-	-	-	-
40.	28.17	2-methyl-7-nonadecene	-	-	-	-	0.82	-
41.	28.20	Methyl 5,9-hexadeca-dienoate	-	-	-	-	1.82	-
42.	28.25	Octadec-9-enoic acid	-	46.28	-	-	-	-
43.	29.33	Eicosane	-	-	-	1.87	-	-
44.	29.34	Cycloeicosane	-	-	-	-	-	11.91
45.	29.36	Nanocosane	-	6.05	-	-	-	-
46.	30.23	Diisooctylphthalate	-	6.16	-	-	33.96	20.21

47.	30.24	Bis(2-ethylhexyl)phthalate	-	-	-	4.00	-	-
48.	30.28	1,4- benzendicarboxylic acid	-	-	-	-	-	6.09
49.	33.56	Cyclotetradecane	-	-	-	-	-	13.75
50.	33.57	(E)-9-eicosene	-	-	-	-	8.50	-
		TOTAL	**95.81**	**98.55**	**96.64**	**98.04**	**93.60**	**96.68**

RT- Retention time

Table 3: Brine Shrimp Toxicity Assay of the Essential Oils *Holarrhena floribunda* and *Crescentia cujete.*

Essential oil	LC_{50} (µg/mL)	UCL (µg/mL)	LCL (µg/mL)
HFL	277.50	465.04	187.14
HFS	257.05	429.12	172.07
HFR	**36.33**	52.04	16.07
CCL	288.76	483.63	194.87
CCS	**10.85**	15.70	4.85
CCR	**16.54**	19.93	3.39

UCL = Upper confidence limit
LCL = Lower confidence limit

The GC-MS of *H. floribunda* leaf essential oil led to the characterization 5 constituents representing 95.81% of the total oil. The oils were dominated by non-terpene compounds, these are; heptadecanolide (11.29%), 1-ethenyloxy-octadecane (8.44%), n-propyl-11-octadecenoate (17.57%), 5-cyclohexadecen-1-one (12.23%) and octadec-9-enoic acid (46.28%), in addition, 8 compounds was characterized in the stem bark oil and dominated mainly by sesquiterpene compounds; τ-muurolol (11.73%) and longifolene (59.77%) while the root bark afforded the characterization of 7 compounds with sesquirosefuran (31.93%), friedelan-3-one(22.85%) (1S,4aS,7R,8aS)-1, 4a-dimethyl-7-(prop-1-en-2-yl) decahydronaphthalen1-ol (17.96%) and hexadecanoic acid (15.86%) being the major compounds.

τ-muurolol, a sesquiterpenoid has been isolated from the marine *Streptomyces* sp. M491(Ding *et al*. 2009). Nii *et al*., 1982 found longifolene and sesquirofuran as the major constituents of the essential oils from *Actinodaphna lancifolia* Meisn. fruits, roots and leaves, Bourgou *et al*., 2010 reported longifolene as a major compound detected in the bioactive essential oil of black cumin while Sousa *et al*, 2012 isolated the triterpene, friedelan-3-one as one of *Chenopodium ambrosiodes* Lineu. and *Kielmeyen neglecta* Sadd. Octadecanoic acid (oleic acid) likewise has been detected in high quantity in the leaf oil of *Prasium majus* from Croatia (Jerkovic *et al*., 2012). All the mentioned constituents were also reported to possess various activities (Ding *et al*., 2009; Nii *et al*., 2008; Bourgou and Legault, 2010; Souza *et al*., 2012; Jerkovic *et al*., 2012.

The characterisation of the leaf oil of *C. cujete* afforded the identification of 15 compounds representing 98.04% and was dominated by a diterpenoid phytol (46.33%). Phytol has been found in appreciable amount in plant essential oils (Jia *et al*., 2008; Aboaba and Udom, 2013). The stem bark oil had 11 components mainly non-terpenes. A high proportion of the oil had diisooctylphthalate (33.96%), (E)-15-heptadecenal (12.77%) and 6-methyl-tridecane (11.46%) while the root oil of *C. cujete* was constituted by 10 compounds representing 96.68% of the entire oil. A sesquiterpene, farnesene (1.48%) was present in the oil. Other compounds were essentially non-terpene compounds. A high quantity of diisooctylphthalate was present in the essential oil of Eaglewood tree (*Aquilaria agallocha* Roxb.) and was suggested as a natural source of the compound as it contained 71.97% of diisooctylphthalate (Bhuiyan *et al*., 2009)

In the present study the brine shrimp lethality of essential oil of leaves, stem bark and root bark of *H. floribunda* and *C. cujete* used in traditional medicine was tested against *Artemia salina* (Brine Shrimp). The result showed that oil from leaves, stem bark and root bark of *H. floribunda* and *C. cujete* was found to be toxic (Table 3) since all the six oils exhibited toxicity level less than 500 µg/mL. The brine shrimp lethality assay represent a rapid, inexpensive and simple bioassay for testing plant oil bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties (Gang *et al*., 2001).

The LC_{50} values of the brine shrimp obtained for essential oil of these medicinal plants as shown in table 3 reveals that the essential oil of *C. cujete* stem bark, *C. cujete* root bark and *H. floribunda* root bark has the most prominent activity with LC_{50} 10.85, 16.54 and 36.33 µg/mL. Therefore, using these oils at higher concentration should be well monitored. The high toxicity can be beneficial in the therapy of some ailments involving cell or tumour growth. It has finally been found out that medicinally active natural products are most toxic to brine shrimp (*Artemia salina*) nauplii (Onocha, 1995).

CONCLUSION

We have characterized the essential oils from the leaf, stem bark and root bark of *H. floribunda* and *C. cujete* from South west Nigeria and to the best of our knowledge, the first report from that region. The abundance of some of the characterised compounds in the studied essential oils may be explored for natural sources of such compounds. The level of toxicity exhibited by some of the plant essential oils as shown above is an indication they could be useful sources of bioactive agents.

CONFLICT OF INTEREST

None declared

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