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Chemical composition and antimicrobial activity of essential oil of *Ocimum kilimandscharicum* (R. Br.) Guerke: A new chemotype

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Abstract
The hydrodistilled essential oils from the flowers and leaves of *Ocimum kilimandscharicum* (R. Br.) Guerke growing in Nigeria were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). Nineteen and thirteen constituents representing 98.0% and 99.6% of the flower and leaf oils were identified, respectively. The major components of the flower oil were methyl eugenol (40.4%), borneol (11.9%) and linalool (10.6%) while the leaf oil consisted mainly of methyl eugenol (53.9%) and γ-cadinene (16.2%). The antimicrobial activities of the oils were assayed against 12 local bacterial isolates and one reference bacterium using agar-disc diffusion and microdilution-broth methods. The results showed that the oils exhibited a wide range of antimicrobial activities. The mean zones of inhibition (IZ) ranged between 7.3 ± 1.5 and 15.1 ± 1.5 mm in the flower; and 9.3 ± 1.7 and 24.7 ± 1.0 mm in the leaf. The minimum inhibitory concentrations (MIC) values varied between 1.25 and 10 mg/mL (flower) and between 0.16 and 10 mg/mL (leaf). A new chemotype of essential oil of *Ocimum kilimandscharicum* is described.

Keywords: *Ocimum kilimandscharicum*, Lamiaceae, essential oil composition, methyl eugenol, antimicrobial activity.

1. Introduction

Ocimum kilimandscharicum (R. Br.) Guerke, (Lamiaceae) is native to East Africa and cultivated in other parts of the world. This species has a strong but less pleasant flavor. It is an aromatic under shrub with pubescent quadrangular branchlets. This plant is easily recognized by its shrubby habit, growing up to 2.44 m tall [1]. It has pale yellow flowers while the leaves are ovate [2]. This plant attracted attention as a source of camphor. In traditional medicine, this plant is widely used for the treatment of various ailments, including colds, coughs, abdominal pains, measles and diarrhea. The leaves have found use in the treatment of congested chest, cough and cold as well as a cure for measles [2].

Extracts of the plant have been shown to possess wound healing [3], antioxidant [4], anti-diarrheal [5], antibacterial [6], antinociceptive [7] and antiamnesic [8] activities. In addition, it has insect repellent [9], mosquito repellent [10, 11] and oviposition deterrence [11] activities. The essential oil was found to be toxic to insect pests [12-14] and possess antibacterial activities [15, 16]. The chemical constituents of *O. kilimandscharicum* populations grown in different parts of the world have been investigated by many researchers. The main compounds of its volatile oils include camphor [5, 12, 17-22, 24, 32-35], α-pinene [34], 1,8-cineole [19, 22, 23, 29, 31-35], linalool [19, 22, 23, 30], limonene [5, 26, 28, 30], eugenol [30, 34], methyl chavicol [30], β-bisabolene [30] and (*E*) -α-bisabolene [30].

In continuation of our studies on the chemical composition of essential oils from aromatic and medicinal plants growing in Nigeria [36], the present investigation reports the chemical composition and antimicrobial activity of essential oils of *O. kilimandscharicum* growing in Lagos, Nigeria.

2. Materials and methods

2.1 Plant collection

Fresh plant materials of *O. kilimandscharicum* were collected from Igando, Alimosho Local Government Area, Lagos State, Nigeria. Botanical identification of the plant material was carried out at the Herbarium of Department of Botany, University of Lagos, Akoka-Yaba, Lagos, Nigeria, where a voucher specimen (LUH 5801) was deposited.

2.2 Extraction of essential oils

Air dried flowers (100 g) and leaves (300 g) of *O. kilimandscharicum* were separately hydrodistillated in a Clevenger-type apparatus for 3 h in accordance with the British Pharmacopoeia specification [37]. The distillate oils were preserved in sealed sample tubes and stored under refrigeration until analysis.

2.3 Gas Chromatography (GC) analysis

GC analysis was carried out on a Hewlett Packard HP 6820 Gas Chromatograph equipped with a FID detector and a DB-5 column (30 m X 0.25 mm id), film thickness was 0.25 µm and the split ratio was 1:25. The oven temperature was programmed from 50 °C (after 2 min) to 240 °C at 5 °C/min and the final temperature was held for 10 min. Injection and detector temperatures were 200 °C and 240 °C, respectively. Hydrogen was the carrier gas. An aliquot (0.5 µL of the diluted oil) was injected into the GC. The peaks were measured by electronic integration. A homologous series of *n*-alkanes were run under the same conditions for determination of retention indices.

2.4 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis of the oil was performed on a Hewlett Packard Gas Chromatography HP 6890 interfaced with Hewlett Packard 5973 mass spectrometer system equipped with a DB-5 column (30m X 0.25 mm id, film thickness 0.25 µm). The oven temperature was programmed from 70- 240 ^{o}C at the rate of 5 ^{o}C/min. The ion source was set at 240 ^{o}C and electron ionization at 70eV. Helium was used as the carrier gas at a flow rate of 1 ml/min. Scanning range was 35 to 425 amu. Diluted oil in *n*-hexane (1.0 µL) was injected into the GC/MS.

2.5 Identification of Components

The components of the oils were identified based on the comparison of their retention indices and mass spectra with those standards, Wiley, 275 library mass spectra database of the GC/MS system and published data [38].

2.6 Antibacterial activity

Ocimum kilimandscharicum essential oils were tested against thirteen local isolates (two Gram-positive, seven Gram-negative strains and three fungal) and one reference bacterial strain obtained from the Department of Microbiology, Lagos State University, Ojo, Lagos and Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria, respectively. These microbes were *Bacillus subtilis*, *Staphylococcus aureus*, *Citrobacter youagae*, *Escherichia coli*, *Escherichia coli* (ATCC 34523), *Klebsiella* spp., *Micrococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Salmonella* spp., *Mucor mucedo* and *Rhizopus stolonifer*. The stock cultures were maintained at 4 ^{o}C in Müeller-Hinton agar (Oxoid, Germany).

2.6.1 Agar disc diffusion

Ocimum kilimandscharicum essential oils were tested for its antibacterial potential by the agar disc diffusion method according to established procedure [39]. The microorganisms were grown overnight at 37 ^{o}C in 20 mL of Müeller-Hinton broth (MHB). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 5 standard (1.0 x 10^{8}) CFU/mL. 90 mm Petri dishes (Merck, South Africa) containing 12 mL of sterilized Müeller-Hinton agar were inoculated with the microbial suspensions. Sterile Whatman No.1 (6 mm) discs, papers were individually placed on the surface of the seeded agar plates and 10 µL of essential oil in dimethylsulfoxide (DMSO) was applied to the filter paper disk. The plates were incubated at 37 ^{o}C for 24 h and the diameter of the resulting zones of inhibition (IZ) was measured. All tests were performed in triplicates. Gentamycin was used as positive control, while hexane and DMSO served as negative controls.

2.6.2 Minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of the oils were determined using 96-well microtiter dilution method as described previously [40]. Bacterial cultures were incubated in Müller-Hinton broth overnight at 37 ^{o}C and a 1:1 dilution of each culture in fresh MHB was prepared prior to use in the micro dilution assay. Sterile water (100 µL) was pipetted into all wells of the microtitre plate, before transferring 100 µL of essential oil in DMSO. Serial dilutions were made to obtain concentrations ranging from 10 mg/mL to 0.078 mg/mL. One hundred µL of bacterial culture of an approximate inoculum size of 1.0 x 10^{8} CFU/mL was added to all well and incubated at 37 ^{o}C for 24 h. After incubation, 40 µL of 0.2 mg/mL, p-iodonitrotetrazolium violet (INT) solution was added to each well and incubated at 37 ^{0}C. Plates were examined after about 30-60 min. of incubation. Microbial growth is indicated by the presence of a reddish colour which is produced when INT, a dehydrogenase activity detecting reagent, is reduced by metabolically active microorganism to the corresponding intensely colored formazan. MIC is defined as the lowest concentration that produces an almost complete inhibition of visible micro-organism growth in liquid medium. Standard antibiotic (gentamycin) and solvent controls (DMSO and hexane) were included in the assay.

2.6.3 Statistical analysis

The mean and standard deviation of three experiments were determined. Statistical analysis of the differences between mean values obtained for experimental groups were calculated as a mean standard deviation (SD) of three independent measurements using Microsoft excel program, 2003. Data were subjected to one way analysis of variance (ANOVA). *P* Values ≤ 0.05 were regarded as significant and *P* values ≤ 0.01 as very significant.

3. Results & Discussion

3.1 Essential oil composition

The yields obtained from the hydrodistillation procedures were 0.23% (v/w) and 0.66% (v/w), calculated on a dry weight

basis respectively for the flower and leaf oils. GC and GC-MS analyses enabled the identification of nineteen and thirteen compounds, respectively, accounting for 98.0% and 99.6% of the total oil contents. Table I indicates the percentage composition and the identities of the components identified in order of their elution on the DB-5 column. Monoterpenes, sesquiterpenes and phenylpropanoids were the main classes of compounds present in both oils.

The major constituents of the oils were similar methyl eugenol (40.4%), borneol (11.9%) and linalool (10.6%) from the flower as well as methyl eugenol (53.9%) and γ-cadinene (16.2%) from the leaf. The other significant compound includes γ-cadinene (7.4%), limonene (4.1%), β-phellandrene (3.4%) and γ-terpinene (3.1%) from the flower oils while borneol (7.2%), caryophyllene oxide (5.5%), linalool (4.5%) and elemol (4.4%) could be found in the leaf. Except for the quantitative amount of linalool, the major compounds of previously investigated samples such as α-pinene, camphene, limonene and camphor were present in much lower amounts in this result while others such as 1, 8-cineole, eugenol, methyl chavicol, β-bisabolene and (*E*)-α-bisabolene were conspicuously absent. Interestingly, the major constituents of the present results, borneol, methyl eugenol and γ-cadinene were not previously reported to be of significant quantities in the essential oils of *O. kilimandscharicum*.

Ocimum kilimandscharicum populations grown in different parts of the world have also been investigated by many researchers (Table 2). In addition to the camphor chemotype [12, 17-22, 24, 32-35], other chemotypes of *O. kilimandscharicum* have also been reported. These were linalool/camphor/1,8-cineole [19, 22,23], camphor/limonene [5, 26, 28, 30], camphor/1,8-cineole [31-33], 1,8-cineole [35], linalool [30], 1,8-cineole/eugenol [30], 1,8-cineole/methyl chavicol/eugenol [30], 1,8-cineole/ β-bisabolene/(*E*)-α-bisabolene [30] and 1,8-cineole/methyl chavicol/β-bisabolene [30] types. In addition, an oil sample was reported to contain multiple amounts of camphor/eugenol/limonene/α-pinene [34]. The present study also identified another chemotype rich in multiple components namely methyl eugenol/borneol/linalool/γ-cadinene for the first time. The presence of methyl eugenol, borneol and γ cadinene in this study and quantitative and qualitative divergence from the previous results from other regions may be due to the geographical, climatic and soil conditions, which in turn may affect the composition and other secondary metabolites of the plant. Also, since the oils have the potential as a source of methyleugenol, it could be used in food and perfume industries [41].

3.2 Antimicrobial activity

The antimicrobial screening of *O. kilimandscharicum* essential oils are summarized in Table 3. The results indicated that the flower oil (IZ; 7.3 ± 1.5 - 15.7 ± 1.5 mm and MIC; 2.5 - 10.0 mg/mL) had weak to moderate activity while the leaf oil (IZ; 9.7 ± 1.2 - 24.7 ± 1.0 mm and MIC; 0.16 – 5.0 mg/mL) displayed better activity against the tested microorganisms. Although, both oils exhibited some inhibitory activities against most of the organisms tested, however, the Gram-negative bacteria *C. youagae*, *Klebsiella* spp., *Proteus* spp., *Salmonella* spp. and a fungus *P. notatum* appeared to be the most resistant organisms. Comparing these results against standard antibiotic (gentamycin, IZ; 13.7 ± 2.1- 13.7 ± 2.1 mm and MIC; 0.31- 2.50 mg/mL), the leaf oil appeared to displayed greater activity against *S. aureus*, *E. coli* and *E. coli* (ATCC 34523) while, the flower oil also flaunted similar action towards *E. coli*, *E. coli* (ATCC 34523) and *Pseudomonas* spp. The present findings are in agreement with previous reports on antimicrobial activity of *O. kilimandscharicum* essential oils [24, 25, 27, 32].

The antimicrobial potential of the studied essential oils of *O. kilimandscharicum* may be attributed to the presence of methyl eugenol which has been reported to possess antimicrobial activity [41-44] as well as a synergy between this compound and other ones (borneol, linalool and γ-cadinene) that were already known to have antimicrobial effects [45-47]. Nevertheless, the presence of minor components such as caryophyllene oxide, elemol, α-eudesmol, limonene and α-pinene might also play a role in the antimicrobial activity of the oil samples [42-44, 48].

3.3 Tables

Table 1: Chemical composition of essential oils of *O. kilimandscharicum*

Compounds [a]	RI (Cal.)	RI (Lit.)	Percentage composition (%)	
			Flowers	Leaves
α-Thujene	935	924	0.4	-
α-Pinene	938	932	1.1	0.1
β-Pinene	976	974	-	0.3
Camphene	951	946	1.2	-
Limonene	1028	1024	4.1	1.2
β-Phellandrene	1032	1025	3.4	-
γ-Terpinene	1062	1054	3.1	-
Linalool	1100	1095	10.6	4.5
Camphor	1141	1141	0.1	0.2
Borneol	1165	1165	11.9	7.2
Methyl eugenol	1411	1403	40.4	53.9
γ-Muurolene	1474	1478	1.9	-
γ-Cadinene	1513	1513	7.4	16.2

δ-Cadinene	1521	1522	-	2.6
Elemol	1546	1548	1.0	4.4
Caryophyllene oxide	1589	1582	1.8	5.5
Viridiflorol	1591	1592	1.0	1.7
Guaiol	1610	1600	2.3	-
α-Eudesmol	1651	1652	2.9	1.8
Bulnesol	1664	1670	2.3	-
Kaur-16-ene	2031	2043	1.1	-
Total			98.0	99.8
Monoterpene hydrocarbons			13.3	1.8
Oxygenated monoterpenes			22.6	11.9
Sesquiterpene hydrocarbons			9.3	18.8
Oxygenated sesquiterpenes			11.3	13.4
Phenylpropanoids			40.4	53.9
Diterpenes			1.1	-

[a] Elution order on DB-5 column; RI (Cal.) = Retention indices relative to C_9-C_{24} *n*-alkanes on the DB-5 column; Literature retention indices; - Not determined.

Table 2: Summary of the major chemical composition of essential oils of *O. kilimandscharicum* from literature

Origin/Part	Biological activity	Major constituents	References
USA (L, Fl)	-	1,8-cineole (10.18-6.38%), linalool (41.94-58.85%) and camphor (17.02-15.82%)	26
‘’ (Sd)	Antioxidant	camphene (7.32%), dl-limonene(13.56%) and camphor (56.07%)	5
‘’	Antibacterial	camphor (63.4-64.9%), limonene (7.9-8.7%), camphene (5.8-6.4%), and γ-terpinene (4.7-0.6%)	28
‘’	-	camphor (46.14%), eugenol (14.43%), 1-8 cineole (7.20%), limonene (13.08%), α-pinene (12.25%) and camphene (7.0%)	38
‘’	-	camphor (45.9%), followed 1,8-cineole (14.6%) and limonene (8.1%) and camphene (5.5%)	35
Canada	-	camphor (78.3%) and 1, 8-cineole (4.4%)	23
‘’	-	linalool (53.1%), camphor (19.3%) and 1, 8-cineole (10.2%)	23
Germany	Antibacterial	camphor (56.9%), 1, 8-cineole (14.63%) and terpinen-4-ol (6.09%)	36
Tanzania	‘’	camphor (52.4%), limonene (7.1) and camphene (5.4%).	29
India	-	camphor (winter 48.9%, summer 58.9%), 1,8-cineole (winter 22.2%, summer 14.8%), and limonene (winter 5.5%, summer 5.6%)	37
‘’	-	camphor (71%)	24
Rwanda	-	1,8-cineole (62.2%)	39
Brazil	-	camphor (35.2%), limonene (16.5%) and camphene (8.12%)	30
India	Antifungal	camphor (66.5%), limonene (6.6%) and camphene (5%)	31
India	Insecticidal	Camphor	16
India (L)	-	linalool (41.94%), camphor (17.0%) and 1,8-cineole (10.18%)	27
India (Fl)	-	linalool (58.85%), camphor (15.82%) and 1,8-cineole (6.38%)	27
‘’	-	camphor (57.87%)	25
‘’	-	camphor (53.89%), limonene (10.5%) and camphene (4.5%)	32
‘’	-	linalool (84.1%), camphor (6.0%), and (*E*)-caryophyllene (2.0%)	34
‘’	-	camphor (43.5–64.9%), limonene (8.7–29.8%), and camphene (0.0–6.4%)	‘’
‘’	-	eugenol (4.5–52.4%), methyl chavicol (7.7–23.3%), β-bisabolene (4.5–22.9%), 1,8-cineole (14.4–20.9%), and (*E*)-α-bisabolene (3.0–10.9%)	‘’
India	-	camphor (64.9%), limonene (8.7%), camphene (6.4%) and (*E*)-β-ocimene (3.0%)	33

L, leaves; Fl, flowers, Sd, seeds; - not known

Table 3: Antimicrobial activity of *O. kilimandscharicum* essential oils

Microorganisms	Flower		Leaf		Gentamycin	
	IZ[a]	MIC[b]	IZ	MIC	IZ	MIC
B. subtilis	13.0 ± 1.5	2.5	15.3 ± 1.7	1.25	23.7 ± 1.5	0.31
S. aureus	13.7 ± 1.5	1.25	24.7 ± 1.0	0. 16	24.3 ± 1.5	1.25
C. youagae	7.3 ± 1.5	10	10.0 ± 1.2	5	20.3 ± 0.6	0.63
E. coli	14.3 ± 1.5	2.5	17.7 ± 1.0	1.25	15.7 ± 1.2	1.25
E. coli (ATCC)	15.7 ± 1.5	1.25	20.3 ± 1.2	0.31	13.7 ± 2.1	2.5
Klebsiella spp.	15.0 ± 1.5	2.5	16.3 ± 1.7	1.25	23.7 ± 1.5	0.31
Micrococcus spp.	12.0 ± 1.5	2.5	10.7 ± 1.0	5	24.3 ± 1.5	1.25
Proteus spp.	8.7 ± 1.5	10	11.7 ± 1.2	5	20.3 ± 0.6	0.63
Pseudomonas spp.	13.3 ± 1.5	5	11.0 ± 1.0	5	15.7 ± 1.2	1.25
Salmonella spp.	9.0 ± 1.5	10	9.7 ± 1.2	5	13.7 ± 2.1	2.5
M. mucedo	12.7 ± 1.5	2.5	13.3 ± 1.7	2.5	23.7 ± 1.5	0.31
P. notatum	10.0 ± 1.0	5	9.3 ± 1.7	10	23.7 ± 1.5	0.31
R. stolonifer	11.0 ± 0.0	2.5	12.7 ± 1.0	1.25	24.3 ± 1.5	1.25

[a] IZ: Inhibition zones diameter (mm) including diameter of sterile disc (6 mm), with values given as mean ± SD (3 replicates); [b] MIC values are given as (mg/mL); ATCC = American Type Culture Collection. [c] Methanolic solutions of Gentamycine - 5µg/ml.

4. Conclusions

The phytochemical analysis of essential oil of *O. kilimandscharicum* led to the delineation of a new chemotype which is hitherto unknown. In addition, the antimicrobial activity of essential oils may suggest the use of the plants and its products as phytopharmaceutical.

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