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Ocimum Gratissimum : the Brine Shrimps Lethality of a New Chemotype Grown in South Western Nigeria

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Strictly as per the compliance and regulations of :

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I. Introduction

Ocimum gratissimum L. (*Lamiaceae*) is known by different people of the world by different local names. It is commonly called Scent leave, 'Nchu anwa' or 'Ahuji' by the Igbo speaking populations of South Eastern Nigeria. 'Effirin-nla' by the Yoruba speaking people of South Western Nigeria and known as 'Daidoya' by the Hausa Speaking populations of Northern Nigeria (Odugbemi, 2006, Saliu et al, 2011). In India it is known by its several vernacular names, 'Vriddhutulsi' (Sanskrit), 'Ram tulsi' (Hindi), 'Nimma tulasi' (Kannada) (Prabhu et al, 2009). In northern Brazil the plant is commonly known as 'Alfavaca-cravo' where it is used in medicine and other culinary purposes (Rabelo et al, 2003).

The nutritional importance of *Ocimum gratissimum* centres on its usefulness as a seasoning because of its aromatic flavour (Okwu, et al, 2006). *O. gratissimum* is an essential ingredient in the local Cuisine popularly known as 'pepper soup' and relished by many in South Western Nigeria. It is an important herbal medicinal plant not only in Nigeria but also in the sub-Saharan Africa. In the southern part of the country, crude aqueous extract of *O. gratissimum* is commonly used in the treatment of epilepsy, high fever and diarrhoea (Effraim et al, 2003). In the Savannah areas, decoctions of the leaves are used to treat mental illness (Akinmoladun, 2007). It is used by the Igbo community of south eastern Nigeria in the management of the baby's cord, to keep the wound surfaces sterile. It is also used in the treatment of fungal infections, fever, cold and catarrh (Ijeh et al, 2005).

Brazilian tropical forest inhabitants use a decoction of *O. gratissimum* in the treatment of digestive disorders (Madeira et al, 2003). In Kenya, it is an important herbal medicine; the leaves are rubbed between the palms and sniffed as a treatment for blocked nostrils (Kokwaro, 1993). They are also used for abdominal pains, sore eyes, and ear infections, for coughs, barrenness, and fever, convulsions, and tooth gargle, regulation of menstruation and as a cure for prolapsed of the rectum (Lexa et al, 2007; Kokwaro, 1993). In India, the whole plant has been used for the treatment of sunstroke, headache, and influenza, as a diaphoretic, antipyretic and for its anti-inflammatory activity (Tania et al, 2006).

Numerous publications have presented data on the composition of the Essential oils of *O. gratissimum*. In early investigations of the Nigerian variety of *O. gratissimum*, the essential oil was found to possess appreciable antibacterial activity against a wide range of organisms (El-Said et al, 1969). Thymol was identified as the major principle which was responsible for the antibacterial activity of *O. gratissimum* but the essential oils of *O. gratissimum* from Europe contain eugenol as the dominant component as well as traces of ocimene and myrcene (Ekundayo, 1986). Analysis of the leaves and flowers volatile oils from different locations in Nigeria confirmed the occurrence of thymol as the main constituent and eugenol was not detected (Sofowora, 1970). However, a recent study of the central Nigerian grown *O. gratissimum* essential oil yielded eugenol (61.9%) as the most abundant compound (Saliu et al, 2011).

The aerial parts of *O. gratissimum* harvested in Togo were composed of thymol (31.79 %), p-cymene (15.57 %) and γ-terpinene (12.34 %) as the major compounds of the essential oil (Koffi et al, 2009).

A Brazillian variety produced eugenol (52.14 %); 1,8-cineole (29.17%), β-seline (5.56%), trans-

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caryophyllene(3.35 %) (Rabelo et al, 2003). Hydro-distilled volatile oils from the leaves of *O. gratissimum* from Meru district in Eastern Kenya were found to be dominated by monoterpenes, eugenol (68.8%), methyl eugenol (13.21%), cis-ocimene (7.47%), trans-ocimene (0.94%), pinene (1.10%) and camphor (0.95%) (Lexa et al, 2007).

Variation of chemical compositions of the essential oil of *O. gratissimum,* eugenol type, was studied for 11 h during the daytime using microwave oven technique, a considerable variation was observed in the eugenol yield, 98% at 12.00 a.m. to 11% at 05.00 p.m. These results show the influence of the solar light on eugenol production and can be useful to indicate the optimal time for collection of the plant (Vasconcelos et al, 1990).

The composition of essential oils from a particular species of plant can differ between harvesting seasons, geographical sources and parts of plant (Burt, S; 2004). Genetic differences in plants of the same species that are otherwise indistinguishable (chemotypes) can also result in widely different essential oil content (Rahimi-Nasrabadi et al, 2009). These may serve to explain the differential medicinal uses of *O. gratissimum* by people of different climes around the world.

Brine Shrimps (*Artemia salina*) is a small aquatic organism which is ideal for toxicity test because it can be cultivated in massive numbers in a limited amount of water and possess a short life span, enabling many experiments to be conducted in a short time. Also, its abundance facilitates replication for statistical analyses (Puente et al, 1992).

It appears that brine shrimp lethality bioassay is predictive of cytotoxicity and pesticidal activity (Pisutthanan et al, 2004). On the basis of these, we investigate the chemical compositions and Brine shrimp lethality of the leaves and seeds essential oils of *Ocimum gratissimum* grown in south western Nigeria.

II. Experimental

a) *Collection of Plant material*

The *Ocimum gratissimum* leaves and seeds were harvested at Apatapiti estate, near the Federal University of Technology, Akure, Ondo state, Nigeria. The identification was done at the Forest Research Institute of Nigeria (FRIN), Ibadan were voucher specimens were deposited.

b) *Isolation of essential oils*

The plant parts of *O. gratissimum* were carefully separated, washed and then subjected to hydro-distillation separately for 2-3hrs using a Clevenger-type glass apparatus, according to European *Pharmacopoeia* (2008) specification. The weight of fresh plant material was 1kg for each plant part. The oil yield was calculated in percentage of volume per weight (v/w) of plant samples.The oil samples were stored in air-tight containers at 0^0C before going for GC-MS analysis without any further treatment

c) *Gas chromatography/mass spectrometry analysis*

The essential oils were analyzed using GC-MS Agilent 6890N GC Coupled with MS-5973-634071 Series. The capillary column type was DB-1MS [30.0m (length) X320.00μm (diameter) X1.00μm (film thickness)]. The carrier gas was Helium at constant flow rate of 1.0ml/min and average velocity of 37cm/s; the pressure was 0.78psi. The initial column temperature was set at 100^0C to the final temperature of 250^0C at the rate of 50C/min, volume injected was 1.0μl and split ratio was 50:1.The total chromatogram was auto-integrated by ShemStation and the constituents were identified by comparison of the GC-MS data with published mass spectral database (NIST02.L) and data from literature.

III. Toxicity test

The cytotoxicity test was conducted on the brine shrimps, *Artemia salina*, using the method of Krishnaraju et al (2005) with some modification.

a) *Hatching of Artemia salina (brine shrimps)*

An improvised aquarium of plastic container with two compartments was used. Holes were created into the divider to allow water circulation and to enable hatched shrimps swim to the side exposed to light. The container (Aquarium) was filled with fresh sea water prior to the introduction of a spatula of brine shrimps to one side which was covered with a booklet as to produce a dark environment which was favoured for hatching, while the other side was left exposed to light. The aquarium was then left for two days until the hatched brine shrimps swam across the divide to the side exposed to light.

b) *Preparation of samples' test solutions*

Stock solution was prepared by emulsifying 20 mg of the essential oils separately in 0.3ml of dimethylsulphoxide (DMSO) and the volume was made up with 1.7ml of fresh sea water to equal 1000 ppm concentration. After this, serial dilution was done to obtain two additional Concentrations of 100ppm and 10 ppm.

c) *Stationing the Brine Shrimps*

Fresh sea water (3.0ml) was transferred into the specimens' vials prepared in triplicates. Then, 0.5ml of each prepared concentrations was introduced into the specimen vials followed by introduction of ten brine shrimps into every specimen vial including the control vial. Finally, every specimen vial was topped up with sea water until it reached 5.0ml. All the vials containing the shrimps were left opened for 24 hours. Finney's probit analysis was used to determine the LC_{50} of each essential oil. Percentage mortality was calculated as number of dead *nauplii* divided by initial number of *nauplii* (10) multiply by 100.Toxicity of the extracts against the brine shrimps were determined by a statistically significant decrease in the survival of brine shrimps exposed to essential oils relative to the survival of shrimps in the control.

IV. Results and Discussions

Hydro-distillation of the leaves and seeds of *O. gratissimum* gave light yellow and orange volatile oils respectively. The essential oil yields of the leaves and seeds were 0.97% and 0.83% respectively. Nine constituents representing 97.09% of total oil from the leaves were identified and Twenty-two compounds representing 98.41% of the total oil were identified from the seeds essential oil (Table 1). The main constituents of the leaves essential oils were found to be γ-terpinene (52.86%), z-tert-butyl-4-hydroxy anisole (13.93%), caryophyllene (10.37%), and P-cymene (7.16%). Other notable representative compounds were identified as thymol (3.65%), γ-selinene (3.45%) and caryophyllene oxide (2.68%). On the other hand, the major constituents of the seeds essential oil were α-pinene (48.19%), 3-tert-Butyl-4- hydroxyl anisole (11.14%) and caryophyllene (10.71%). Other constituents were α- selinene (4.00%), caryophyllene oxide (3.45%), p-cymenene (3.86%) and thymol (3.29%). Six compounds were found to be common constituents of the leaves and the seeds essential oils of *O. gratissimum*. They were caryophyllene, caryophyllene oxide, p-cymenene, γ-selinene, tert-butyl-4-hydroxy anisole and thymol.

Artemia salina nauplii were found to be highly susceptible to the essential oils of *O.gratissimum* (Table 2). At concentration of 1000 ppm for all the extracts, the ten brine shrimps used in the triplicate determination died after 24hrs which equal100% mortality of the *nauplii.* Mortality of 93-97% was recorded for 100ppm concentrations. The susceptibility of the *nauplii* at 10ppm ranges between 55-86% mortality.

Table1 : Chemical compositions of the essential oil of leaves and seeds of *Ocimum gratissimum*.

	Chemical compounds	Retention times (Min.)	Percentage composition (%) Leaves	Seeds
1	γ-Terpinene	2.20	52.86	-
2	α-Pinene	2.22	-	48.19
3.	p-cymenene	22.29	7.16	-
4	(Z)-β-Terpineol	2.34	-	0.92
5	1,3,8-p-Menthatriene	2.42	-	0.51
6	(4)-Terpineol	2.92	-	1.39
7	Thymol methyl ester	3.45	3.65	3.29
8	Unbellulone	3.91	1.57	1.23
9	Methoxy mesitylene	4.72	-	1.11
10	m-Eugenol	5.34	-	0.96
11	α-Cubebene	5.87	-	0.67
12	β-Elemene	6.15	-	2.11
13	Caryophellene	6.66	10.37	10.71
14	1-Ethyl-3-(propen-1-yl)adamantane	6.94	-	1.03
15	α-caryophyllene	7.17	1.42	1.70
16	2-tery-Butyl-4-hydroxyanisole	7.80	13.93	-
17	3-tert-Butyl-4- hydroxyanisole	7.81	-	11.14
18	γ-Selinene	8.01	3.4	-

19	α –Selinene	8.04	-	4.0
20	α –Panasinsen	8.34	-	0.64
21	Caryophyllene oxide	9.45	2.68	3.45
22	2-Methylene-6, 8, 8-trimethyl Tricyclo [5.2.2.0](1,6)undecan-3-ol	11.87	-	0.60
23	Monoamide, N-(3-acetylphenyl) benzene-1, 2-dicarboxylic acid	17.51	-	0.99
24	3, 4-Xylenol	18.43	-	0.65
25	Trimethyl-8-(-1-methylethyl) -2,4(1H,3H)-phenanthrenedione	22.93	-	0.93
26	2-(4-Hydroxy-3-methoxyphenyl) -3,7-dimethoxy-4H-chromen-4-one	22.95	2.96	-
	Total		97.04 %	96.22%

Table 2 : Toxicity of the essential oils using brine shrimps (*Artemia salina*) lethality assay.

Essential oil	No. of dead Shrimps; conc.1000ppm	%Mortality	No. of dead Shrimps; Conc. 100ppm	%Mortality	No. of dead Shrimps; Conc. 10ppm	%Mortality	LC_{50} µg/ml
OGL	10,10,10	100	9,10,9	93.3	7,7,6	66.7	3.6031
OGS	10, 10, 10	100	10,10,8	93.3	6,6,4	55.5	8.1588

Key: OGL= *O. gratissimum* leaves' essential oil, OGS= *O. gratissimum* seeds' essential oil.

VI. Summary and Conclusions

The major constituents of the leaves' essential oils of *O. gratissimum* were γ-terpenene, caryophyllene, p-cymene, Z-tert-butyl-4-hydroxyanisole while the seeds essential oils of *O. gratissimum* yielded α-pinene, caryophyllene, 3-tert-Butyl-4-hydroxyanisole and α-selinene as the major products in this study. The genus *Ocimum* has been reported to yield essential oils of varying chemical compositions around the globe. Guenthor, (1948) grouped *O. gratissimum* into thymol and eugenol chemotypes based on literature reports on the essential oils of *O. gratissimum* around the world which suggested thymol, and or eugenol as the major constituents. Previous reports from Nigeria (Sofowora, 1970; Iwalokun et al, 2001; Saliu et al, 2011) were in agreement with this classification. However, many other chemotypes have been reported in literature around the world subsequently. Chemotypes characterized by high contents of geraniol (Charles et al, 1992), methyl cinnamate (Fun et al., 1990), ethylcinnamate (Ali et al, 1968), eugenol/methyl eugenol (Lexa et al, 2007) and citral (Hegnauer, 1967) have been reported. Accordingly, the extracted essential oil in this study could be classified as the γ-terpenene/ α-pinene chemotype. This chemotype is similar in chemical compositions to a Togo variety (Koffi et al, 2009) which had γ-terpenene (12.34%) as one of the major constituent of the leaves' essential oils of *O. gratissimum.* Keita et al. (2000) have also reported thymol (46%), p-cymeme (12%) and γ-terpene + trans-sabinene hydrate (17%) for *O. gratissimum* in the Republic of Guinea. The occurrence of tert-butyl-4-hydroxy anisole in the leaves and seeds essential oils of this chemotype is of particular health benefits; tert-butyl-4-hydroxy anisole is known to prevent or delay the propagation of harmful radicals in the living cells.The essential oils of *O. gratissimum* (leaves and seeds) were lethal to *Artemia salina* which is predictive of cytotoxicity and pesticidal activity.

VII. Acknowledgements

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