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Larvicidal Potential of Leaf Extracts and Purified Fraction *Ocimum Gratissimum* against *Culex Quinquefasciatus* Mosquito Larva

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Abstract: ***Purification and structural identificationof the mosquito larvicidal compounds present in Ocimum gratissimum leaf extracts against Culex quinquefasciatus mosquito larvae. The larvicidal potential of the aqueous, ethanol, ethtylacetate, n-hexane and purified fractions of O. gratissimum were tested against the early fourth and third instars larvae of C.quinquefasciatus Mosquito species. Larvicidal toxicity assay were performed using different concentrations and larval mortality was observed after 12 hr, 24 hr and 48 hr of treatment respectively. Thin layer and Column chromatography were used to purify the fraction with the highest larvicidal activity while FTIR and GC-MS were used for structural identification of the phytoconstituents. The result of phytochemical studies revealed the presence of sterols, flavonoids, alkaloids and saponins in O. gratissimum n-Hexane extract. The effective larvicidal activity was observed in O. gratissimum n-hexane extract against C. quinquefasciatus mosquito larvae with percentage mortality of 96% at 25ppm after 24hr whereas LC_{50} and LC_{90} 2.57 and 2.52ppm respectively. Following the bioactivity-guided fractionation,the active constituent was found in Fraction three of O. gratissimum n-Hexane extract(F3) with percentage mortality of 100% at 100ppm after 24hr whereas LC_{50} and LC_{90} of (1.28 and 4.28ppm) respectively.Isolated from O. gratissimum n-Hexane fraction(F3) was characterized as caryophylleneoxide in band C by spectroscopic analyses. The Lethal concentration LC_{50} of the isolated caryophylleneoxide was 2.21ppm and with percentage mortality of 100%. These work showed that Caryophyllene oxides might be a potent larvicidal agent for mosquito control.***

Keywords: Larvicidal, Ocimum gratissimum, *Culex quinquefasciatus*, Spectroscopic, Lethal Concentration**.**

1. Introduction

Mosquito control is an important integral component in the efforts directed towards the reduction and or elimination of the health burdens of malaria, lymphatic filariasis, yellow fever, dengue, chikungunya and encephalitis. Mosquitoes are responsible for the world's most serious vector borne diseases.These diseases are globally transmitted to humans by several species of mosquito [1] and these diseases are manifested in over 700 million individuals living in 100 countries annually [2].The use of several generations of synthetic organophosphate, organochloride and carbonate insecticides have jointly succeeded in partial reduction of the pest problems attributed by mosquitoes [3].There are several atrocious consequences of synthetic insecticide usage on the environment, water, soil, air and genomic stability as well as human and animal health generally.Thus, there is the need to search for alternative pesticides from the rich diversity of plant forms which as selective bio pesticides could be used as control agents at the larval stage of the vector.

*Ocimum gratissimum*is one of such plants belonging to the Lamiaceae family [4], commonly called Wild basil, Dai doya in Hausa, Efinrinajase in Yoruba and Nchuanwu in Igbo. Farmers in northern Nigeria indigenously use various parts of the plants to protect cereals and legumes against pest damage during storage [5], [6]. Local use of the plant leaf shows that it has mosquito repellant properties[7], [8] and in India whole plant preparations are used to treat stomach ache, sunstroke, headache and influenza [9], [10]. The seeds have laxative properties and are also used in the treatment of gonorrhea (4). The essential oil is applied against fever,inflammations of the throat, ears, eyes, stomach pain, diarrhoea and skin diseases [10]. Therefore, the present work aimed at purification and structural identification of mosquito larvicidal agent in controlling mosquitoes as a disease vector.

2. Material and Methods

2.1 Collection of plants

The plant leaves of *Ocimum gratissimum*were harvested around Ahmadu Bello University Zaria, Area "A" staff quarters, Nigeria with voucher no. 1285 and deposited at the herbarium unit, Department of Biological science, Ahmadu Bello University, Zaria, Nigeria.

2.2 Plant collection/ extraction preparation

Dried powder (100g) was dissolved in 1000ml of distilled water, ethanol, ethylacetate and n-hexane respectively. Extractions were carried out using cold maceration. The filtrates were concentrated using rotary evaporator at 45^0C and stored till required for use.

2.3 Culturing of Mosquito larvae

An uninfected strain of *C. quenquefaciatus* was harvested around Ahmadu Bello University Zaria, water works, Main Campus and reared in the laboratory by standard rearing procedures. About 150 larvae were reared in plastic trays until the adults emerged. Adults were maintained in a screen cage (60×30×30 cm) at 27±2˚C and 75-85% Relative humidity [11]. The females were fed with blood every alternate day whereas the males were fed with 10% glucose solution soaked on cotton pad, which were hung in the

middle of the cage. A beaker with strips of moistened filter paper was kept for oviposition. The eggs were put into plastic tray containing tap water to hatch. Crackers biscuit, brewer's yeast and algae in the ratio of 3:1:1 was served as food for the emerging larvae. The third and fourth in stars larvae were used for the assay.

2.4 Determination of larvicidalproperties

Bioassay was performed following the WHO guidelines [11, 13]. Twenty five (25) third and fourth instar larvae in triplicates were used. Different concentrations of the extracts in increasing order of 6.25 ppm, 12.5 ppm, 25 ppm, 50 ppm, 100 ppm and 1000 ppm. The larvae were transferred by using a camel hair brush into the test solution each in 100 ml of tap water in boiled and cooled tap water (chlorine-free water). Control replicate of dimethylsulphuroxide (DMSO) and Dichlorvos in 100ml of distilled water respectively were tested simultaneously and the larvicidal mortality was determined at 12 hr, 24 hr and 48 hr post exposure. Corrected Abbott formula was used where mortality in the control is greater 15%[11]. Prick test was used to determine mortality and moribund larvae were considered death.

2.5 Purification of metabolites

The TLC result showed that Ocimum gratissimum n-hexane extract expressed six (6) bands with distinctiveR_f value ranging between 0.44 and 0. 83 respectively. About 3.0g ethanol extract of *O. gratissimum* were purified using column chromatography to identify the compound(s) responsible for the larvicidal activity. A total of 41 fractions (50ml/15min) were collected. Each of the fractions was spotted on TLC plate. The developed TLC plate was allowed to dry and was viewed under UV radiation (λ=254 nm). The plate was further sprayed with 20% sulfuric acid in alcohol solutions and dried at 110°C for 1min [14]. These two procedures enabled the fractions to be pooled together into 7 main fractions each based on the pattern and R_f values of the spots on the TLC plate. Fractions with the same R_f value were pooled and the intermediate pooled fractions were further subjected to Larvicidal bioassay. The active fraction against the mosquito was further separated by prep-TLC.

2.6 Identification of phytochemical constituent by GC-MS spectroscopy

Identification of phytochemical constituent showing larvicidal potentials was subjected to GC-MS analysis. The metabolites were dissolved in their respective solvents (HPLC grade). About (1µl) were injected into a RTX-5 column (60m X 0.25mm, film thickness 0.25µm) of GC-MS (model GC-MS-QP-2010 plus, Shimadzu Make). Helium was used as carrier gas at a constant column flow rate 1.58 ml/min at 108kpa inlet pressure. The final confirmation of constituents was made by computer matching of the mass spectra of peaks with the National Institute of Science and Technology (NIST) libraries 2005 mass spectral database at the National Research Institute for chemical Technology, Zaria Kaduna State, Nigeria.

2.7 Statistical analysis

Results were expressed as percentage mortality and lethal concentration (LC_{50} and LC_{90}) using Abbott formulae [15] and probit analysis [16], [17]. One way ANOVA were used to test for significant difference in larval mortality among various concentration of *O. gratissimum*.

3. Result

3.1 Percent mortality and LC_{50} of different Solvent extract of Ocimum gratissimum leaves against *C. quenquefaciatus* Mosquitoes Larvae

Percentage mortality and lethal concentration of crude aqueous, ethanol, ethylacetate and n-hexane extracts of *O. gratissimum* leaves against *C. quenquefaciatus* mosquito larvae in Table 1, revealed that N-hexane extract presented 100 per cent mortality at 1000ppm after 48 hours and 96% mortality at 25ppm after 24hr of treatment which indicated to be the best solvent for extraction of biolarvicidal active compound. The LC_{50} of the n-hexane extract was the lowest at 2.52ppm and LC_{90} of about 30.51ppm followed by Ethanol extract with LC_{50} of 3.29ppm and LC_{90} of 55.25ppm. On the other hand aqueous extract had the highest LC_{50} at 36.173ppm with very poor mortality. Hexane extract had the lowest LC_{50} with a relatively more toxic characteristic and was considered to be the best solvent system for the extraction of Larvicidal active principle.

Table 1: Mortality and lethal concentration of different extracts of O. gratissimum against C. *quenquifaciatus* Mosquito Larvae

Extract	Percent mortality (%)							Lethal Conc. (ppm)	
	Conc. (ppm)	6.25	12.5	25	50	100	1000	LC_{50}	LC_{90}
Aque	12hr	0	0	0	0	0	8	36.1	320
	24hr	0	0	0	20	9	13		
	48hr	0	0	0	27	45	49		
EtOH	12hr	0	0	8	19	16	52	3.6	55.3
	24hr	36	47	79	80	80	80		
	48hr	36	75	78	80	80	80		
EtAC	12hr	0	0	5	15	16	40	7.7	27.4
	24hr	4	4	8	16	16	44		
	48hr	4	4	8	16	16	44		
n-Hex	12hr	0	32	40	12	32	59	2.5	30.5
	24hr	48	72	96	83	80	95		
	48hr	64	83	99	99	95	100		

No. of replicates 3 @ 25 larvae/replicate, Aque=aqueous extract, EtOH= ethanol extract, EtAC= ethylacetate extract, n-Hex= n=hexane extract.

3.2 Percentage mortality and lethal concentration of OGnHE-F3 Ocimum gratissimum n-hexane fractions against C. Quinquefasciatus larvae

The combined fractions were subjected to a comprehensive larvicidal study at different concentrations, of 6.25ppm, 12.5ppm, 25ppm, 50ppm, 100ppm. The fraction (OGnHE-F3) was found to be active larvicidal agent against C. quinquefasciatus larvae. The percentage mortality of OGnHE-F3 showed maximum activity of 100% at 50ppm

and an effective larvicidal toxicity of 100% at 100ppm after 24hr and 12hr of treatments against C. quinquefasciatus larvaerespectively. Lethal concentration of the effective n-hexane fraction at LC50 and LC90were found to be 1.49ppm and 4.28ppm respectively. Moderate activity was found in OGnHE-F2 and OGnHE-F5 while the OGnHE-F1 and OGnHE-F6 showed low activity even at higher concentrations.

Table 2: Larvicidal activity of OGnHE-F3 Ocimum gratissimum n-hexane partially purified Fraction against Culex quenquifaciatus after 12hr and 24 hr treatments

Fraction	*Percent mortality (%)*						*Lethal Conc(ppm)*	
	Conc.(ppm)	*6.25*	*12.5*	*25*	*50*	*100*	*LCa50*	*LCb90*
Ctrl	12hr	40	68	95	100	100	2.04a	5.42
	24hr	44	80	100	100	100	(0.61-3.47)	(3.27-23.80)
F1	12hr	0	0	7	7	13	17.51f	83.28
	24hr	0	0	7	7	20	(7.6-18.10)	(17.4-331.9)
F2	12hr	0	20	20	40	46	3.79b	9.15
	24hr	0	26	33	40	60	(3.20-4.86)	(6.50-92.7)
F3	12hr	20	66	87	87	100	1.49a	4.28
	24hr	26	80	93	100	100	(1.03-2.89)	(3.20-7.51)
F4	12hr	0	13	20	20	23	7.39e	29.92
	24hr	0	13	13	20	33	(5.0-29.31)	(12.46-97.62)
F5	12hr	0	6	20	53	27	4.34c	15.24
	24hr	0	20	33	60	33	(3.43-6.74)	(8.80-64.53)
F6	12hr	0	6	20	40	40	5.07d	13.54
	24hr	0	7	26	40	40	(4.07-8.05)	(8.38-48.79)

No. of replicates 3 @ 25 larvae/replicate, Ctrl= standard control group, F1-6 various fraction obtained by column chromatography.

Table 3: Major Compounds present in the leave bioactive fraction F3 of O. gratissimum n-hexane extract by GCMS

PN	RT	Compound Name	Area%	MW (Da)
1	10.68	Thymol	24.34	150
2	14.6	Caryophyllene oxide	10.91	220
3	17.83	Hexahydrofarnesyl acetone	7.67	268
4	22.9	Stearic acid,methyl ester	4.88	298
5	25.71	Δ8-Tetrahydrocannabinol	6.56	314
6	27.06	dl-2-Ethylhexyl chloroformate	4.28	192
7	27.75	Di-n-octyl phthalate	41.36	390

3.3 Characterization of thecompound(s) in the Bioactive Fraction F3 by FTIR and GC-MS Spectroscopy

The FTIR indicates the presence of C-H i.e. (aromatic, alkyl group and methylene) bond of strong intensity, C=O (carboxylic acids derivatives, aldehydes and saturated aliphatic group and O-H (Phenol or Alcohol) bond of strong intensity. While Table 3, GCMS result of fraction three (F3) revealed the presence of seven compounds with name and area in percentage are shown in Table 3. These include Thymol (24.34%), caryophylleneoxide (10.91%) and di-n-octyl phthalate (41.36%) was found as the 3 major components and the minor compounds include Hexahydrofarnesyl acetone (7.67%), stearic acid, methyl ester (4.88%), DELTA.8-tetrahydrocannabinol (6.56%) and dl-2-ethylhexyl chloroformate (4.28%) in terms of abundance.

Table 4: Probit analysis of Lethal Concentrations of isolated band B OGnHE-F3 against C. *quenquefaciatus*

Band	*Compounds*	*Regression equation*	*LC50*	*LC90*	*Mortality (%)*
Band B	Caryophyllene oxide	y =0.04x+12.27	1.49	4.28	100
Dichlorvos	Control	y =7.40x-0.42	2.04	3.46	100

Number of replicates @ 25 larvae/replicate(r=3) at each concentration; LC_{50}, lethal concentration for killing 50 percent of the treated larvae.

4. Discussion

Currently there is resurgence of interest in plant derived compounds for developing commercially ecofriendly insecticides. This phytochemicals have been shown to have insecticidal property against several egg, larvae, pupae, nymph and adult of insect. The high percentage mortality observed in *O. gratissimumn*-Hexane leaf extract was in the ability of *n*-hexane in extracting non polar compounds which could be responsible for the oily active ethnobotanicals application as mosquito larvicide and repellent property, this statement was also acknowledged by Egunyomi [18].

Reports revealed that several phytochemicals act as toxicants which generally kill different life stages of the insect while various other interfere with growth and metamorphosis [19], [20].

Evaluation of the mosquito larvicidal potential of the aqueous, ethanol, ethylacetate and n-hexane leaf extract of *O. gratissimum* against the fourth-instar larvae mosquito (*Culex quinquefasciatus*) vector of malaria, lymphatic filariasis. The concentration of a substance needed to kill half of a population at a specific time of observation is refered to as lethal concentration. This present study showed that*O. gratissimum*, *n*-hexane extract had low LC_{50} value with high larvicidal activity against the vector. The corresponding $LC_{50}$and LC_{50} values of 2.5ppm and 30.5ppm were similar to that ofacetone extract of Ocimum sanctum reported to have a toxic effect against the larvae of *Spodopteralitura*, *Aedesaegypti* and *Culex quinquefasciatus*[21], [22]. This toxicity could be attributed to the presence of many groups

of phytochemical compounds such as triterpenoids, alkaloid and sterol in n-hexane extract which also correlates also to the assertion of Kishore*et al*, [23], [24] and [25] whose finding showed that neem kernel powder have high toxic effect on feeding and survival of different pest species and insects under Laboratory condition. Similarly the development of the different intermediate stages (Larval-Pupal, Pupal-Adult) and the formation of abnormal pupae and adult are also an indication of the insect growth regulation properties of the plant extracts which were indicative of malfunctioning of endocrine system. Formation of the malformed larval-pupal intermediate is also reported to be physiological effect of Neem [26].

The methanol extract of *Ocimum canum* and the acetone extract of *Ocimum sanctum* were reported to have a toxic effect against the larvae of *Spodopteralitura*, *Aedesaegypti*and*Culex quinquefasciatus* [27, 28], the methanol extract of dried root powder of Rhinacanthusnasutus was tested against the larvae of *A. aegypti* and *C. quinquefasciatus* [29] and the methanol extract of *R. nasutus* was reported to have a pesticidal effect against the larvae of *S. litura*[29].

The larvicidal properties of the fractions obtained from the column chromatographic purification of OGnHE fraction are Thymol, Caryophyllene oxide and Di-n-octylphthalate these could be the compounds responsible for the larvicidal activity. Previous studies of assessment of the larvicidal potentials of thymol derivatives on anopheles mosquitos by Jack and others [30, 31] where appreciable mortality. In the present study mortality in the larvae was found to be in a dose dependent fashion.
This could be attributed partly to decreased solubility of these derivatives as the molecular mass increases. Spreading thymol derivatives over water surface obstruct the breathing of the larvae thereby suffocating them or acting as poison. Glycerin and 6-Pentyl-5, 6-dihydro-2H-pyran-2-one groups are better electron withdrawing groups which are electron releasing groups previously reported [31, 32].The detection of Thymol ion of this compound is in consonance with the work reported by other scientists in different plant species viz., *Psoraleacorylifolia* Linn [32, 33].

This study demonstrated that the *O. gratissimum* n-Hexane leaf extract containedCaryophyllene oxide as potent and effective mosquito larvicide.

5. Other Recommendations

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