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Towards the use of extracts from *Plectranthus glandulosus* (Lamiaceae) and *Callistemon rigidus* (Myrtaceae) leaves to indoor-spray (control) Malaria and other arboviral diseases vector mosquitoes

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Abstract
The extensive use of synthetic mosquitocides has affected our ecosystem, environment, and health of living beings. Mosquitoes also gained resistance toward those chemicals. Botanicals, which showed their activities against various mosquito species, may be exploratory sources. This study aimed at evaluating the toxicity of different solvent extracts and essential oils against three adult mosquito vectors. Four solvents *viz* hexane, chloroform, ethyl acetate and methanol fractions from the crude methanol extract and essential oil of *Plectranthus glandulosus* and *Callistemon rigidus* were used against female *Culex quinquefasciatus*, *Anopheles gambiae* and *Aedes aegypti* adults. The standard Centres for Disease Control and Prevention Bottle Bioassay for mosquito adulticidal agents was performed. Under the laboratory conditions, the mortality was observed 24 h post-treatment for extracts and fractions and 10 minutes for essential oils and WARRIOR®. All the extracts and fractions of both plants showed good adulticidal activity except chloroform, ethyl acetate and methanol fractions of *Callistemon rigidus* which exhibited no toxicity whereas essential oils, very good mosquito adulticidal activity. The LC_{50} values of *P. glandulosus'* essential oil were 62.6, 48.23 and 45.5 µg/250ml while those of *C. rigidus* were 412.7, 273.1 and 334.3 µg/250ml against *Cx. quinquefasciatus*, *An. gambiae* and *Ae. aegypti*, respectively. No dead mosquito was registered in negative controls and no mosquito was found alive in positive controls (WARRIOR®). These results give way to conclude that eco-friendly extracts from Cameroonian *C. rigidus* and *P. glandulosus* leaves are good potentials which may replace the synthetic WARRIOR® for the control of malaria, yellow fever, dengue and filariasis vector mosquitoes.

Keywords: Mosquitoes, adulticidal activity, *Plectranthus glandulosus*, *Callistemon rigidus*, fractions, essential oil

Introduction
Mosquitoes are insects which suck blood from their hosts (mammals) to mature their eggs. They lay eggs into any places containing stagnant water [1]. Once they emerge from their breeding sites, they start biting their hosts, both indoors and outdoors [2]. While hunting, their bites cause sleepless nights and days because of their noise, skin scratching and irritations [3].

In addition, they transmit to humans deadly diseases like malaria (mostly in Africa), haemorrhagic dengue fever, zika, yellow fever and other arboviral diseases [4]. *Culex quinquefasciatus Anopheles gambiae* and *Aedes aegypti* are the principal transmitters of these diseases. By biting with their proboscis, they transmit the virus, parasite, bacterium or helminth from an infected individual to a healthy person [4].

To limit the transmission of the mosquito borne diseases, measures have been put in place. The use of biological control, ovicides, larvicides, pupicides and adulticides has been developed, targeting all life stages of mosquitoes. Unfortunately, those carriers succeed to escape all those control methods, finding themselves biting their hosts and so transmitting diseases [5].

Researchers are currently using a technique known as indoor residual house spraying (IRS) which is to spray a lethal dose of insecticide onto walls and roofs of houses. The insecticide adheres to the mosquito once it has rested on a sprayed surface [5].

Synthetic insecticides have been used over decades to control mosquito vectors [6]. They are very effective, but they have shown numerous adverse effects on humans, environment and non-target organisms. Mosquitoes have also developed resistance against those insecticides as they are made of a single active principle [7, 9].
These concerns have pushed scientists in search of alternative. Insecticides of plant origin are now recognized as safer alternatives. Essam *et al.* [10]. have listed various plant species with their numerous mosquitocidal activities.
The present project investigated on the toxicity of *P. glandulosus* and *C. rigidus* leaf extracts and essential oils from Cameroon against *Cx. quinquefasciatus*, *An. gambiae* and *Ae. aegypti*. This is the first report on extracts from both plants against mosquito adults.

Materials and Methods

Source of mosquito species

Cx. quinquefasciatus, *An. gambiae* and *Ae. aegypti* were reared in the insectarium of the Faculty of Pharmaceutical Sciences, Agulu; Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The emerged adults were under the temperature of 29±3°C and 82±4% of relative humidity. The adults were fed with 10% sugar solution. 40 cm x 40 cm cages were used.

Harvest of plant materials and plant extractions

The fresh leaves of *P. glandulosus* and *C. rigidus* were harvested from 6:00 to 11:00 am in Cameroon, Adamawa region, Ngaoundere and dried in room temperature. The dried leaves were then ground in powder, kept in opaque containers and moved to Nigeria where the extractions and bioassays were carried out. Once in Nigeria, the powders were stored in a freezer set at -8°C until needed.
To extract and fractionate the plant materials, the method adopted by Okoye and Osadede [11] was used. The crude extracts were obtained by soaking 700 g of each plant's powder in methanol, stirring them thrice a day and filtering them by using a Whatman Nº 1 filter paper. The obtained crude extracts (9.96 and 27.28% for *P. glandulosus* and *C. rigidus*, respectively) were then adsorbed in silica gel and eluded in succession with hexane, chloroform, ethyl acetate and methanol to obtain HF=10.9%, CF=13.6%, EAF=12.9% and MF=32.9%, respectively for *P. glandulosus*; and HF=8.2%, CF=11.4%, EAF=12.4% and MF=37.6%, respectively for *C. rigidus*.
The extraction of the essential oils was submitted to hydro-distillation in a full glass Clevenger apparatus to give reddish-yellow oil (0.2% w/w) for *P. glandulosus* and very importantly white-clear oil (0.7% w/w) for *C. rigidus* [12]. The anhydrous sodium sulphate was used to remove the traces of water from the oil. All the extracts were rolled with aluminium foil and stored in a freezer (-8°) until used.

Coating the test bottles

The CDC bottle bioassay was followed up [13]. Briefly, 250 ml bottles, thoroughly washed and dried, were used. Crude extracts, fractions and essential oils were serially diluted with acetone. Four bottles for a phytochemical, one for positive control and one for negative control (acetone) were used for each insecticide and replicate. Each bottle and its lid were labelled. After adding the product in the bottle, its lid was immediately put on and the bottle was evenly coated. At the end of the coating, all the bottles were kept open in a dark cupboard overnight to get well dried. The following day, after ensuring that the bottles were dry, the lids were put on and the bioassays started. 1 ml disposable syringes were used and each product had its labelled syringe.

Performing the bottle bioassays

After the bottles were removed from cupboard and their lids put on, they were lined up. 25 female mosquitoes aged 4-7 days were gently collected from the stock with a mouth aspirator and introduced in each bottle. The mosquito introduction process started with negative control bottles and once the mosquitoes were introduced into the bottle, the lid was immediately pot on back. All mosquitoes were fed on 10% sugar solution. 125-1000 µg/250 ml bottle for essential oil, 1000µg/250 ml for WARRIOR® (100% DDVP: 2,2-dichlorovinyl dimethyl phosphate) and 1250-10000 µg/250 ml bottle for crude extracts and fractions. Four replicates were performed and each replicate consisted of six bottles (five treated and one negative control). After the requisite time passed, mortality was scored after 10 min for essential oil and WARRIOR® and 24 hours for crude extracts and fractions under ambient conditions (27±2 °C and 79±2%).

Statistical analysis

Data were subjected to ANOVA followed by Duncan Multiple Comparison Tests (DMCT) to compare mean percentage mortality between dosages. Results with $p<0.05$ were considered to be statistically significant. Probit analysis of Finney [14] was used to calculate the LC_{50} and LC_{90} values as well as their confidence limits by using SPSS 17.0 for Windows® software.

Results

Crude extracts, fractions and essential oil from *C. rigidus* and *P. glandulosus* showed their adulticidal efficacy increasing with the increase of concentrations and varied from one mosquito species to the other.
Both extract and fractions of *P. glandulosus* achieved 100% mortality except MF against all the target mosquito species at higher concentration which was 10000 µg/250 ml (Table 1). The HF was the most active fraction exhibiting 10.7, 64 and 53.3% mortality at lower concentration (1250 µg/250 ml) with the LC_{50} values of 2745.5, 950.2 and 1203.3 µg/250 ml against *Cx. quinquefasciatus*, *Ae. aegypti* and *An. gambiae*, respectively (Table 4). The same observation was made with MCE and HF of *C. rigidus* displaying 100% mortality against all the mosquito species at higher concentration which was still 10000 µg/250 ml (Table 2). However, CF, EAF and MF presented no mortality at all concentrations. The HF even at 1250 µg/250 ml, still induced mortality rates of 6.7, 40 and 21.3 (Table 2), having the LC_{50} values of 2540.5, 1923.9 and 1961.5 µg/250 ml against *Cx. quinquefasciatus*, *Ae. aegypti* and *An. gambiae*, respectively (Tables 5). The essential oil of both plant materials demonstrated very good toxicity against all the three mosquito species just after 10 minutes of exposure. *P. glandulosus*, even at 125 µg/250 ml (lowest concentration), ravaged almost all the exposed female mosquitoes. This was demonstrated by the inflicted lethal rates of 74.7, 86.7 and 85.3% against *Cx. quinquefasciatus*, *Ae. aegypti* and *An. gambiae*, respectively (Table 3). The calculated LC_{50} were as low as 62.6, 45.5 and 48.2 µg/250 ml against *Cx. quinquefasciatus*, *Ae. Aegypti* and *An. gambiae*, respectively (Table 4). The synthetic insecticide (WARRIOR®) killed all the exposed female mosquitoes after 10 minutes and no dead was observed in negative control.

Table 1: Mosquito adulticidal activity of *P. glandulosus* extracts against three mosquito species.

Extracts	Conc. (µg/250ml)	Mean ± Std. Deviation of the mortality (%)		
		Cx. quinquefasciatus	***Ae. aegypti***	***An. gambiae***
Methanol crude extract	1250	12 ± 4^{a}	9.3 ± 2.3^{a}	16 ± 6.9^{a}
	2500	54.7 ± 6.1^{b}	64 ± 8^{b}	74.7 ± 8.3^{b}
	5000	76 ± 12^{c}	89.3 ± 4.6^{c}	93.3 ± 8.3^{c}
	10000	100^{d}	100^{d}	100^{d}
	F value	84.8***	216.8***	93.8***
Hexane fraction	1250	10.7 ± 2.3^{a}	64 ± 8^{a}	53.3 ± 6.1^{a}
	2500	37.3 ± 6.1^{b}	84 ± 8^{b}	78.7 ± 6.1^{b}
	5000	86.7 ± 10.1^{c}	97.3 ± 4.6^{c}	94.7 ± 2.3^{c}
	10000	100^{d}	100^{c}	100^{c}
	F value	145.8***	21.7***	65.8***
Chloroform fraction	1250	0^{a}	16 ± 8^{a}	10.7 ± 4.6^{a}
	2500	29.3 ± 4.6^{b}	46.7 ± 4.6^{b}	52 ± 4^{b}
	5000	58.7 ± 4.6^{c}	84 ± 8^{c}	74.7 ± 6.1^{c}
	10000	100^{d}	100^{d}	100^{d}
	F value	512.4***	114.6***	230.9***
Ethyl acetate fraction	1250	0^{a}	0^{a}	0^{a}
	2500	17.3 ± 6.1^{b}	12 ± 4^{b}	29.3 ± 6.1^{b}
	5000	38.7 ± 4.6^{c}	44 ± 8^{c}	44 ± 8^{c}
	10000	100^{d}	100^{d}	100^{d}
	F value	389.4***	299.8***	208.6***
Methanol fraction	1250	0^{a}	0^{a}	0^{a}
	2500	0^{a}	0^{a}	0^{a}
	5000	0^{a}	6.7 ± 4.6^{b}	4^{a}
	10000	18.7 ± 6.1^{b}	16 ± 4^{c}	38.7 ± 4.6^{b}
	F value	28***	18.4**	197.9***

**: $p < 0.01$; ***: $p < 0.001$.

Table 2: Mosquito adulticidal efficacy of *C. rigidus* extracts against three mosquito species.

Extracts	Conc. (µg/250ml)	Mean ± Std. Deviation of the mortality (%)		
		Cx. quinquefasciatus	***Ae. aegypti***	***An. gambiae***
Methanol crude extract	1250	0^{a}	0^{a}	0^{a}
	2500	29.3 ± 6.1^{b}	9.3 ± 2.3^{b}	19.7 ± 4^{b}
	5000	68 ± 6.9^{c}	42.7 ± 6.1^{c}	76 ± 8^{c}
	10000	100^{d}	100^{d}	100^{d}
	F value	269.5***	574.8***	320.2***
Hexane fraction	1250	6.7 ± 2.3^{a}	40^{a}	21.3 ± 2.3^{a}
	2500	52 ± 10.5^{b}	53.3 ± 4.6^{b}	68 ± 8^{b}
	5000	89.3 ± 6.1^{c}	77.3 ± 6.1^{c}	93.3 ± 4.6^{c}
	10000	100^{c}	100^{d}	100^{c}
	F value	138.4***	143.8***	168.3***
Chloroform, Ethyl acetate and Methanol fractions	1250, 2500, 5000 & 10000	No death registered	No death registered	No death registered

***: $p < 0.001$.

Table 3: Mosquito adulticidal effect of *P. glandulosus* and *C. rigidus* essential oils against three mosquito species.

Species	Conc. (µg/250ml)	Mean ± Std. Deviation adulticidal mortality (%)	
		P. glandulosus	*C. rigidus*
Ae. aegypti	125	86.7 ± 6.1^{a}	13.3 ± 2.3^{a}
	250	94.7 ± 2.3^{b}	37.3 ± 6.1^{b}
	500	100^{b}	54.7 ± 4.6^{c}
	1000	100^{b}	100^{d}
	F value	11.1**	251.2***
An. gambiae	125	85.3 ± 2.3^{a}	10.7 ± 2.3^{a}
	250	97.3 ± 2.3^{b}	46.7 ± 4.6^{b}
	500	100^{b}	78.7 ± 4.6^{c}
	1000	100^{b}	100^{d}
	F value	55.1***	379.6***
Cx. quinquefasciatus	125	74.7 ± 6.1^{a}	0^{a}
	250	78.7 ± 8.3^{a}	29.3 ± 6.1^{b}
	500	93.3 ± 6.1^{b}	46.7 ± 4.6^{c}
	1000	100^{b}	100^{d}
	F value	11.9**	360.9***

**: $p<0.01$, ***: $p<0.001$.

Table 4: Probit-analysis for mosquito adulticidal activity of *P. glandulosus* extract/fractions and essential oil against 3 hazardous mosquito species.

Species	Extracts/ Fractions/ Essential oils	Lethal Conc. 50% (Confidence Limits) in µg/250ml)	Lethal Conc. 90% (Confidence Limits) in µg/250ml
Ae. aegypti	MCE	2300.5 (1878-2765.8)	4524.5 (3621.6-6633.4)
	HF	950.2 (318.1-1383.6)	2930.4 (2137.8-5842.1)
	CF	2515.6 (2012-3088.5)	5701.7 (4403.6-8911.4)
	EAF	4703.7 (3986.7-5612.5)	8190.3 (6648.2-11873.1)
	MF	23741.6 /	77196.4 /
	EO	45.5 (-)	153.8 (-)
An. gambiae	MCE	1972.1 (1587.1-2373.9)	3867 (3097.4-5730.1)
	HF	1203.3 (613.3-1642.4)	3598.1 (2656.3-6795.9)
	CF	2706.5 (2160-3331.5)	6288.5 (4830.1-9918.1)
	EAF	4198.1 (-)	8608.1 (-)
	MF	11419.1 (9242.9-22006)	20748.3 (14054.2-114238)
	EO	48.2 (-)	150.1 (-)
Cx. quinquefasciatus	MCE	2603.4 (2064.6-3209.8)	6126.3 (4696.6-9715.9)
	HF	2745.5 (2260.4-3323.8)	5564.2 (4399.4-8274.9)
	CF	3820.7 (-)	7414.2 (-)
	EAF	4693.6 (-)	8762.2 (-)
	MF	16159.1 (-)	32012.2 (-)
	EO	62.6 (3.11-119.6)	347.9 (222.17-1077.5)

MCE: Methanol Crude Extract, HF: Hexane Fraction, EAF: Ethyl Acetate Fraction, MF: Methanol Fraction, EO: Essential Oil.

Table 5: Probit-analysis for mosquito adulticidal activity of *C. rigidus* extract/fractions and essential oil against 3 hazardous mosquito species.

Species	Extracts/ Fractions/ Essential oils	Lethal Conc. 50% (Confidence Limits) in µg/250ml	Lethal Conc. 90% (Confidence Limits) in µg/250ml
Ae. aegypti	MCE	4834.8 (4116.5-5742.5)	8146.7 (6666.1-11681.4)
	HF	1923.9 (1245.2-2557.9)	6902.8 (4778.8-14813)
	EO	334.3 (-)	898.2 (-)
An. gambiae	MCE	3697.8 (3148.9-4346.8)	6076.5 (5036.8-8499.8)
	HF	1961.4 (1546.8-2387.7)	4138.4 (3258.7-6362.7)
	EO	273.1 (220.5-334.1)	605.4 (469.9-933.5)
Cx. quinquefasciatus	MCE	3597.5 (3011.9-4300)	6677.5 (5381.8-9686.2)
	HF	2540.4 (2105.4-3040.2)	4807.4 (3872.4-3872.4)
	EO	412.7 (-)	838.75 (-)

MCE: Methanol Crude Extract, HF: Hexane Fraction, EO: Essential Oil.

Discussion

Through our literature survey, there is no published report available on the mosquito adulticidal activity of *P. glandulosus* and *C. rigidus*. However, the essential oil of *P. glandulosus* showed its adult toxicity on stored products pests [15, 16].

Many researchers have also previously published papers on different plant extracts against numerous adult mosquito species. For example, Rajamohan and Marimuthu [17] reported that hexane, ethyl acetate, benzene, chloroform and methanol extracts of *Coccinia indicia* were effective against *Cx. quinquefasciatus* adults. Additionally, hexane, ethyl acetate, benzene, chloroform and methanol extracts of *Eclipta alba* and *Andrographis paniculata* showed their prove of toxicity against *Anopheles stephensi* adults [18].

Moreover, Govindarajan and Sivakumar [19] reported that hexane, ethyl acetate, benzene, chloroform and methanol leaf extracts of *Cardiospermum halicacabum* registered good results on *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* adults. In the same vein, hexane, benzene, ethyl acetate, acetone and methanol leaf extracts of *Acalypha alnifolia* were found very active against adults of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* [20].

Furthermore, the essential oil from *Lantana camarai* was very toxic against five mosquito species, namely *An. stephensi*, *An. culicifaciesi, An. fluviatilis, Ae. Aegyptii and Cx. quinquefasciatusi* [21].

Comparing the results presented in this project to the results of previous works may not be an easy task. In fact, the efficacy of a plant extract depends on the extraction process, the type of solvent used, the concentration of metabolites present, the geographical situation of the plant, the part used, the mosquito species used for the bioassay and the condition in which the test was carried out [22].

Though the mode of action of this project's different extracts was not assessed, Rattan [23] showed that photochemicals act on cholinergic, GABA, mitochondrial and octopaminergic systems of the insect. In addition, he highlighted the behaviours of insects towards

The mode of action of these leaf extracts, fractions and essential oils on mosquito adult are not known, but the symptoms observed in adult mosquitoes were similar to those caused by nerve poisons *i.e*., excitation, convulsion, paralysis and death. The same observations caused by phytochemicals were also observed by Choochote *et al*. [24] and Dua *et al*. [21].

Conclusion

The findings of the present investigation revealed that the crude extracts, fractions and essential oils of *P. glandulosus* and *C. rigidus* possess remarkable adulticidal activity against medically important vector mosquitoes. These extracts especially the essential oil of *P. glandulosus* may be used directly as mosquito adulticidal agent for indoor residual house spraying. Studies to confirm this hypothesis in field condition are underway in a project to formulate a natural mosquito spray using the essential oil of *P. glandulosus* in our laboratory in order to replace the synthetic WARRIOR®.

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