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Larvicidal and Pupicidal Activities of *Plectranthus glandulosus* and *Callistemon rigidus* Leaf Essential Oils against Three Mosquito Species

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Abstract

Objective: To determine the mosquito larvicidal and pupicidal activities of Cameroonian *Plectranthus glandulosus* and *Callistemon rigidus* leaf essential oils against 4th instar larvae and early pupae of *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus*.

Methods: Twenty five early 4th instar larvae and early pupae of *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* were exposed to various concentrations ranging from 12.5-500 ppm and 2000 ppm for DDVP. The standard protocol of WHO 2005 was followed up under laboratory conditions. Larval and pupal mortalities were observed 24 h post-exposure. Probit analysis was used to compute the LC_{50} and LC_{90} values.

Results: The essential oil of the two plants showed significant larval and pupal toxicities against all the target mosquito species. *P. glandulosus* caused LC_{50} values of 2.66, 7.37 and 43.16 ppm against *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* larvae, respectively and 27.22, 22.60 and 104.75 ppm against *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* pupae, respectively. *C. rigidus* displayed LC_{50} values of 66.67, 99.61 and 176.81 ppm against *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* larvae, respectively and 50.95, 47.63 and 307.19 ppm against *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* pupae, respectively.

Conclusion: These results suggest that the Cameroonian *P. glandulosus* and *C. rigidus* leaf essential oils can be used as an ideal eco-friendly approach which may replace DDVP, the synthetic chemical, for vector control programs.

Keywords *Plectranthus glandulosus*; *Callistemon rigidus*; Essential oil; Mosquito larvicide; Mosquito pupicide

Introduction

Mosquitoes are vectors of many ailments worldwide. They are responsible of lymphatic filariasis (*Cx. quinquefasciatus* Say), yellow fever, dengue (*Ae. aegypti* Linn.) and malaria (*An. gambiae* Giles) (Ndione et al., 2013). WHO revealed this: "Behind the statistics and graphs lies a great and needless tragedy: malaria still takes the life of an African child every minute". In Cameroon, *An. gambiae* is the only mosquito species causing thousands of death every year especially to children under five and pregnant women (WHO, 2012a). To illustrate this, residents of Cameroon's Far North Region have been gripped by malaria outbreak where 302 persons died in September 2013 alone (Mulango, 2013). In addition, according to WHO (2012b), Cameroon occupied the sixth position all over the world in yellow fever (YF) cases. An outbreak of YF has been reported in the North Region of Cameroon (Demanou et al., 2012). Moreover, cases of Chikungunya Virus have been detected among the citizens living within Douala and Yaoundé in Cameroon in 2006 (Peyrefitte et al., 2006).

To avoid the worst in malaria death and the outbreak of yellow fever, dengue, filariasis or chikungunya cases, it is very urgent to intervene in tackling those

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mosquito borne diseases rather than tackling the diseases since it is known that "prevention is better than cure" (Manzoor et al., 2013). To control those vectors, chemical insecticides such as Temephos, S-methoprene, Monomolecular films, Spinosad, Bti, etc. have been used to reduce mosquito larvae or pupae and as such prevent diseases (Brattsten and Hamilton, 2012). Despite their effectiveness against mosquitoes, chemical insecticides have some disadvantages such as pollution, toxicity of residues to the non-target organisms and resistance (Devine and Furlong, 2007). To find new modes of action and to develop active agents based on natural plant products, efforts are made to develop phytochemical insecticides (Gokulakrishnan et al., 2013). In 1991, Sukumar et al. (1991) reviewed the use of natural products derived from 344 different plant species to control mosquito populations. Thus, the plant derived products have received increased attention from scientists and nowadays more than 2000 plant species have already been screened as potent insecticides providing possible lead candidates to replace synthetic chemical insecticides for controlling mosquito in aquatic stages (larvae and pupae) (Essam et al., 2005; Anupam et al., 2012). In this regard, a survey of the literature on insecticidal properties of essential oils from the year 2004 onwards indicates that essential oils from about 90 plant genera belonging to 38 plant families were reported to have toxic properties against mosquito larvae (Mann and Kaufman, 2012) and pupae (Gokulakrishnan et al., 2013).

Plecthrantus glandulosus Hook f (Lamiaceae) is a plant which is adapted nearly to all types of areas and altitudes (Abdel-Mogib et al., 2002). This plant is found in West Africa flora (Ngassoum et al., 2001) and also in Cameroon flora (Nukenine et al., 2013). Still in Cameroon, it is plant whose leaves are commonly used to protect stored grains and flour (Nukenine et al., 2013; Goudoum et al., 2013).

C. rigidus R. Br. (Myrtaceae) is known in folk medicine for its anticough, antibronchitis effects and its essential oils exhibited antifungal activity against *Phaeoramularia angolensis* (Jazet et al., 2009) and *Aspergillus flavus* (Dongmo et al., 2010).

In Cameroon, previous works showed the richness of the two plant materials in volatile constituents quantitatively and qualitatively (Nukenine et al., 2007; Goudoum et al., 2012; Praveen et al., 2012).

Therefore, the aim of this work was to evaluate the larvicidal and pupicidal potential of *P. glandulosus* and *C. rigidus* essential oils against *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* 4th instar larvae and early pupae.

1 Results

The yield of the essential oils extraction was 0.22% (w/w) for *P. glandulosus* and 0.72% (w/w) for *C. rigidus*.

The larvicidal and pupicidal activities of *P. glandulosus* and *C. rigidus* were found to be mosquito species dependent and plant dependent.

The larvicidal toxicity of *P. glandulosus* essential oil is against 4th instar larvae of *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciattus* after 24h of exposure showed that *Ae. aegypti* was the most susceptible species among the three target mosquito species in this study with a LC_{50} and LC_{90} values of as small as 2.66 and 8.71 ppm, respectively (Table 1). Statistically, there was no difference among all the concentrations ($F= 2.28$; $p>0.05$). *An. gambiae* came in second position to register a mortality rate of 76% with the smallest concentration (12.5 ppm) and total suppression from 50 ppm; displaying LC_{50} and LC_{90} values of 7.37 and 25.55 ppm, respectively. However, *Cx. quinquefasciattus* was the most resistant mosquito species to record no mortality at the lowest concentration and all tested larvae were killed at concentrations of 100 and 150 ppm with LC_{50} and LC_{90} values of 43.16 and 67 ppm, respectively.

The results of mosquito larvicidal activity of *C. rigidus* essential oil against 4th instar larvae of *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* 24 h after treatment are summarised in (Table 2). From these results, *Ae. aegypti* was also the most susceptible mosquito species among those targeted in this study achieving LC_{50} and LC_{90} values of 66.67 and 124.39 ppm, respectively and causing 32% mortality at the lowest concentration of was 50 ppm. As for *An. gambiae* which still came in second position

Table 1 Larvicidal activity of *P. glandulosus* essential oil against 4th instar larvae of *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciattus* 24 h post exposure

Targeted mosquito species	Conc.(ppm)	MM±SD (%)	LC_{50} (LCL−UCL) (ppm)	LC_{90} (LCL−UCL) (ppm)	χ^2
Aedes aegypti	12.5	94.67±6.11[a]	2.66	8.71	0.23
	25	100.00±0.00[a]	(−)	(−)	
	50	100.00±0.00[a]			
	100	100.00±0.00[a]			
	150	100.00±0.00[a]			
	F value	2.28ns			
Anopheles gambiae	12.5	76.00±6.92[a]	7.37	25.55	2.91
	25	81.33±6.11[a]	(1.17−11.98)	(17.65−51.43)	
	50	100.00±0.00[b]			
	100	100.00±0.00[b]			
	150	100.00±0.00[b]			
	F value	24.62***			
Culex quinquefasciatus	12.5	0.00±0.00[a]	43.16	67.00	0.31
	25	6.67±2.31[b]	(37.07−50.34)	(56.27−92.40)	
	50	64.00±6.93[c]			
	100	100.00±0.00[d]			
	150	100.00±0.00[d]			
	F value	667.15***			

Note: MM±SD (%): Mean of mortality ± standard deviation (%); MM±SD within a column followed by the same letter do not differ significantly at P= 0.05 (Duncan's test); ***: p<0.001; LC_{50} and LC_{90}: Lethal Concentrations able to kill 50% and 90% of larvae, respectively; ppm: parts per million; LCL: Lower Confidence Limit; UCL: Upper Confidence Limit; (−): no Confidence Limit estimated; ns: not significant; χ2: Chi−squared; Number of replicates: 4

Table 2 Larvicidal activity of *C. rigidus* essential oil against 4th instar larvae of *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* 24 h post exposure

Targeted Mosquito species	Conc.(ppm)	MM±SD (%)	LC_{50} (LCL−UCL) (ppm)	LC_{90} (LCL−UCL) (ppm)	χ^2
Aedes aegypti	50	32.00±6.92[a]	66.67	124.39	2.14
	100	70.67±4.61[b]	(53.38−78.48)	(104.29−163.63)	
	150	97.33±4.61[c]			
	200	100.00±0.00[c]			
	250	100.00±0.00[c]			
	F value	144.41***			
Anopheles gambiae	50	14.67±1.15[a]	99.61	179.42	5.46
	100	46.67±4.61[b]	(59.50−136.06)	(132.08−435.97)	
	150	69.33±6.11[c]			
	200	100.00±0.00[d]			
	250	100.00±0.00[d]			
	F value	333.24***			
Culex quinquefasciatus	50	0.00±0.00[a]	176.81	227.57	9.77
	100	3.67±0.57[a]	(42.29−327.94)	(185.29−3099.46)	
	150	13.33±2.30[b]			
	200	69.33±6.11[c]			
	250	100.00±0.00[d]			
	F value	702.41***			

Note: MM±SD (%): Mean of mortality ± standard deviation (%); MM±SD within a column followed by the same letter do not differ significantly at P= 0.05 (Duncan's test); ***: p<0.001; LC_{50} and LC_{90}: Lethal Concentrations able to kill 50% and 90% of larvae, respectively; ppm: parts per million; LCL: Lower Confidence Limit; UCL: Upper Confidence Limit; χ2: Chi-squared; Number of replicates: 4

in term of susceptibility, 14.67% mortality was achieved with the concentration of 50 ppm and utterly suppressed the exposed larvae in 100 and 200 ppm getting LC_{50} and LC_{90} values of 99.61 and 179.42 ppm, respectively. *Cx. quinquefasciatus* was still the most resistant mosquito species against the poisonous effect of *C. rigidus* essential oil. They presented the highest mortality rate of 69.33 and 100% only with 100 and 200 ppm, respectively.

The toxicity results of *P. glandulosus* essential oil against early pupal stages of *Ae. aegypti*, *An. gambiae* and *Cx quinquefasciatus* 24 h of exposure clearly indicated that the oil was toxic against all the three mosquito species tested (Table 3). *An. gambiae* was the most susceptible mosquito pupae displaying LC_{50} and LC_{90} values of 22.60 and 140.53 ppm, respectively and killing 61.33% of exposed pupae at lower concentration (25 ppm) and 100% with 300 ppm. *Ae. aegypti* recorded mortality of 54.67% with the lowest concentration (25 ppm) and 100% from 200 ppm. *Cx quinquefasciatus* showed higher LC_{50} and LC_{90} values of 104.75 and 225.05 ppm, respectively registering no mortality at lower concentration of 25 ppm but total suppression with 300 ppm.

Table 3 Mosquito pupicidal activity of *P. glandulosus* essential oil against *Ae. aegypti*, *An. gambiae* and *Cx quinquefasciatus* pupae 24 h post exposure

Mosquito species used	Conc.(ppm)	MM±SD (%)	LC_{50} (LCL–UCL) (ppm)	LC_{90} (LCL–UCL) (ppm)	χ^2
Aedes aegypti	25	54.67±4.61[a]	27.22	93.42	2.87
	50	62.67±4.61[b]	(15.24–37.32)	(67.96–168.35)	
	100	90.67±5.11[c]			
	200	100.00±0.00[d]			
	300	100.00±0.00[d]			
	F value	86.40***			
Anopheles gambiae	25	61.33±6.11[a]	22.60	140.53	3.92
	50	65.33±4.61[a]	(7.64–36.21)	(91.76–344.88)	
	100	76.00±5.00[b]			
	200	96.00±4.00[c]			
	300	100.00±0.00[c]			
	F value	33.40***			
Culex quinquefasciatus	25	0.00±0.00[a]	104.75	225.05	3.93
	50	10.67±4.61[b]	(86.20–126.00)	(179.33–320.06)	
	100	54.67±4.61[c]			
	200	76.00±3.00[d]			
	300	100.00±0.00[e]			
	F value	254.17***			

Note: MM±SD (%): Mean of mortality ± standard deviation (%); MM±SD within a column followed by the same letter do not differ significantly at P= 0.05 (Duncan's test); ***: $p<0.001$; LC_{50} and LC_{90}: Lethal Concentrations able to kill 50% and 90% of pupae respectively; ppm: parts per million; LCL: Lower Confidence Limit; UCL: Upper Confidence Limit; χ2: Chi-squared; Number of replicates: 4

The results presented in Table 4 showed the mortality of the early pupal stages of *Ae. aegypti*, *An. gambiae* and *Cx quinquefasciatus* by *C. rigidus* leaf essential oil after 24 h of exposure. Percent mortality gradually increased with increasing oil concentrations. With the LC_{50} of 47.63 ppm, the oil killed all the exposed *An. gambiae* pupae at 300 ppm. The same LC_{50} values of 50.95 and 307.19 ppm were observed against *Ae. aegypti* and *Cx quinquefasciatus*, respectively Neither larvae nor pupae survived against DDVP, the conventional mosquitocide.

2 Discussion

Essential oil from various plants has been found to be toxic against different mosquito species in the field of vector control (Mann and Kaufman, 2012; Asgar, 2013).

Table 4 Mosquito pupicidal activity of *C. rigidus* essential oil against *Ae. aegypti*, *An. gambiae* and *Cx quinquefasciatus* pupae 24 h post exposure

Targeted Mosquito species	Conc.(ppm)	MM±SD (%)	LC_{50} (LCL−UCL) (ppm)	LC_{90} (LCL−UCL) (ppm)	χ^2
Aedes aegypti	25	18.67±4.61[a]	50.95	129.91	2.44
	50	50.67±4.11[b]	(39.70−63.19)	(99.42−201.06)	
	100	73.33±5.32[c]			
	200	100.00±0.00[d]			
	300	100.00±0.00[d]			
	F value	140.89***			
Anopheles gambiae	25	44.00±5.58[a]	47.63	389.46	7.38
	50	46.67±5.11[a]	(−)	(−)	
	100	57.33±3.11[a]			
	200	72.00±3.00[b]			
	300	100.00±0.00[c]			
	F value	31.48***			
Culex quinquefasciatus	100	0.00±0.00[a]	307.19	503.61	6.30
	200	18.67±4.61[b]	(189.80−437.66)	(378.09−821.03)	
	300	44.00±3.00[c]			
	400	61.33±4.61[d]			
	500	100.00±0.00[e]			
	F value	211.32***			

Note: MM±SD (%): Mean of mortality ± standard deviation (%); MM±SD within a column followed by the same letter do not differ significantly at P= 0.05 (Duncan's test); ***: p<0.001; LC_{50} and LC_{90}: Lethal Concentrations to kill 50% and 90% of pupae respectively; ppm: parts per million; LCL: Lower Confidence Limit; UCL: Upper Confidence Limit; (−): no Confidence Limit estimated; χ2: Chi−squared; Number of replicates: 4

Essential oils extracted from plants are biodegradable with non-residual effects on the biological environment due to their rapid volatility. Hence, an attempt was made in the present study to investigate the larvicidal and pupicidal potentials of two locally available plants in Cameroon, *P. glandulosus* and *C. rigidus* in the control of mosquitoes.

Previous works in Cameroon showed the richness of the two plant materials in volatile constituents quantitatively and qualitatively (Nukenine et al., 2007; Goudoum et al., 2012; Praveen et al., 2012). These phytoconstituents may be responsible for the mosquito larvicidal and pupicidal potentials claimed in this study.

The characterization of *P. glandulosus* essential oil showed a high percentage of oxygenated monoterpenes (58.6% and 84.6% for fresh and dried leaves, respectively) represented by cis-piperitone oxide (3.0% and 35.1%), trans-piperitone oxide (0.5% and 12.6%), fenchone (30.8% and 21.6%) and piperitenone oxide (10.9% and 6.0%). The main monoterpene hydrocarbons were terpinolene (25.2% and 7.7%), limonene (3.2% and 1.7%) and myrcene (2.2% and 1.6%). The sesquiterpene derivatives were found in a very low percentage (<2.5%), represented mainly by germacrene D (1.4% and 1.0%) (Ngassoum et al., 2001). In the same vein, the volatile constituents of the dried powdered leaf essential oil from the same plant revealed the presence of thymol (3.7%), terpineolene (8.2%), fenchone (18.3%), piperitone epoxide (17.7%), piperitone oxide (8.9%), piperitone (1.2%), diosphenol (2.5%) and cis-piperitone (19.5%) (Nukenine et al., 2007). Goudoum et al. (2012) analysis showed 15 compounds with highest rate of Fenchone (29.8%) and α-terpinolene (28.3%). Tatsadjieu et al. (2008) found 23 compounds among which the main constituents were found to be β-thujone (30.8%), terpinolene (25.2%) and piperitenone oxide (10.9%). Yaouba et al. (2012) analysis showed that the main components found in *P. glandulosus* leaves oil were terpinolene (30.8%), fenchone (13.2%), terpene 4-ol (11%) and piperitenone oxyde (8%).

In *C. rigidus*, monoterpenoids and sesquiterpenoids have been identified as ingredients of the essential oil in the leaves, seeds and fruits. Specifically, triterpenoids (α-amyrin, betulinic acid and oleanolic acid), tannins (pyragallol, catechol and β-sitosterol) were found in arial parts (Jirovetz et al., 1997; Praveen et al., 2012). Flavonoids (3'4'7-trihydroxy flavonol, 3'4'7-trihydroxy flavone, 3'4'7-trihydroxy flavonol-3-glycoside and 3'4'7-trihydroxy flavone-7-galactoside) and monotriterpenes (γ-terpinene, α-terpeneol, α-pinene, 1,8-cineol and limonene), melaleucin and melaleucin acetate have also been reported to be constituents of the leaves (Praveen et al., 2012).

The results of the present study are compared with earlier reports. The bioactivity of ten plant oils, Cedar wood (*Cedrus atlantica* (Endl.) Carrière), Citronella (*Cymbopogon nardus* (Linn.) W. Watson), Clove (*Myrtus caryophyllum* Spreng), Eucalyptus (*Eucalyptus globulus* Labill. (Myrtaceae)), Lemon grass (*Cymbopogon flexuosus* (Steud) Wats), Orange (*Citrus sinensis* (Linn.), Nutmeg (*Myristica fragrans* Houtt.), Palmarosa (*Cymbopogon martini* Roxb.), Pine (*Pinus radiate* D. Don) and Tulsi (*Ocimum sanctum* Tulsi) were tested against the 3rd instar larvae of *Ae. aegypti*. Larval mortality was observed after 24 h. Among the plant oils tested, orange oil exhibited the highest larvicidal activity with LC_{50} of 85.93 ppm, followed by Palmarosa with 88.78 ppm, Tulsi with 92.48 ppm and Nutmeg oil with 93.62 ppm (Tennyson et al., 2013). In addition, five essential oils from various parts of five plant species i.e. *Acorus calamus* Linn. (Acoraceae), *Mentha arvensis* Linn. (Lamiaceae), *Ocimum basilicum* Linn. (Lamiaceae), *Saussrea lappa* C.B. Clarke (Asteraceae) and *Cymbopogon citrates* (DC.) Stapf (Apocynaceae) were investigated for their larvicidal property against *Ae. aegypti* and *Cx. quinquefaciatus* larvae. The highest larvicidal activity was observed in the essential oil of *O. basilicum* against *Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} values of 75.35 ppm and 92.30 ppm, respectively (Manzoor et al., 2013). Moreover, five major components in essential oil from Pogostemon cablin (Blanco) Benth. (Lamiaceae) (â-patchoulene, á-guaiene, ã-patchoulene, á-bulnesene and patchouli alcohol) were tested for pupicidal activity against three medically important human vector mosquitoes at 100 mg/L. Among the five compounds tested, patchouli alcohol was found to be the most effective for pupicidal activity provided 28.44, 26.28 and 25.36% against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*, respectively (Gokulakrishnan et al., 2013).

Essential oils are presumed to interfere with basic metabolic, biochemical, physiological and behavioural functions of insects (Mann and Kaufman, 2012). Though the mechanism of action (site effect) of the two essential oils was not established in this study, yet earlier, Ndione *et al*. (2013) reported the larvicidal and cytopathologic effects of Suneem 1% (neem: *Azadirachta indica* A. Juss (Meliaceae)) on *An. gambiae* and *Cx. quinquefasciatus* larvae. In the control group, within 24 to 48 h, in photonic microscopy level, an epithelial cell layer was noted with a brush border well developed, an adipose and muscle normal tissues. The epithelial cells contain various organelles such as mitochondria, dictyosoms, golgi apparatus, a network endoplasmic reticulum and granular. In the treated group, a gradual disappearance of cellular organelles and significant vacuolation was observed. Cytoplasm was less homogeneous. Ruiz et al. (2004) found a significant vacuolation, swollen nuclei and elongated epithelial cells of the mosquito larvae treated with essential oil. These epithelial cells were disrupted at the apical region with vesicle formation, lysis and leakage of cytoplasm material into the gut lumen. In addition, it is also known that the mosquito larvae and pupae breathe through spiracles located on the 8th abdominal segment and therefore must come to the surface frequently to breathe. The oils block the spiracles, resulting in lack a respiratory siphon (asphyxiation) and death (Kaufmann and Briegel, 2004; Rotimi et al., 2011).

In conclusion, the findings of the present study reveals that essential oils from the leaves of Cameroonian *P. glandulosus* and *C. rigidus* can be effectively used as potent mosquito larvicides and pupicides. Application of these oils could be very useful to reduce the larvae and pupae of those vectors borne-diseases breeding in wide variety of containers, ranging from watering cans and discarded plastic bags to ground depressions and

blocked roof gutters especially in the Far North Region of Cameroon. This would offer an eco-friendly and less expensive way which may replace DDVP, the conventional chemical, to reduce the problem of these mosquitoes known as "enemy number one of mankind woldwide", especially where the examined plants are largly available and used in Cameroon. However, carrying out studies on mode of action in mosquito physiology, persistance, synergistic effect of the two plant essential oils and field trial in the nearest future still need to be done.

3 Materials and Methods

3.1 Collection of plant materials

The fresh leaves of *P. glandulosus* Hook f (Lamiaceae) and *C. rigidus* R. Br. (Myrtaceae) were collected in October 2011 (6:00 am-11:00 am GMT) in Ngaoundere (latitude 7° 22' North and longitude 13° 34' East, altitude of 1100 masl), located in the Adamawa region (plateau), Cameroon. The plants were less than one-year old and only the green leaves were harvested. These plants were identified for confirmation at the National Herbarium of Cameroon, where voucher specimens were deposited with the following voucher number: 18564/SRF/CAM and 41168HCN for *C. rigidus* and *P. glandulosus*, respectively. Leaves were dried at room temperature of (25±3)℃ and (81±2)% RH, and then ground in powder using electric grinder until the powder passed through a 0.4 mm mesh sieve. The powder was stored in opaque containers inside a refrigerator at -4 ℃ and transported by road in February 2012 to Faculty of Pharmaceutical Siences, Agulu, Nnamdi Azikiwe University, Awka, Anambra state, Nigeria by road where the experiments were carried out and then stored in a freezer at -4℃ until needed.

3.2 Test organisms

The larvae of *Ae. aegypti* and *Cx. quinquefasciatus* were collected from WHO/National Arbovirus and Vector Research Centre Enugu, Enugu state, Nigeria. The larvae of *An. gambiae* were collected from Awka market, Anambra State, Nigeria inside the gutter and identified to WHO/National Arbovirus and Vector Research Centre Enugu, Enugu state, Nigeria. All the different genera larvae were then brought into the insectarium in the Faculty of Pharmaceutical Sciences, Agulu, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria where they were reared. The larvae of *Ae. aegypti* and *Cx. quinquefasciatus* were fed with chicken feed (grower) mixed with fish feed in 3:1 ratio. Those of *An. gambiae* fed on ground chicken feed (grower), yeast and fish feed in 3:1:2 ratios. On every alternate day, the water from the culture bowl was changed carefully until pupation. The feeding was continued till the larvae transformed into the pupal stage. The larvae and early pupae were maintained at (28 ± 4)℃, (80±5)% RH and under 12 Light: 12 Dark photoperiod cycles.

3.3 Extraction of essential oils

The method adopted by Diksha et al. 2012 and Aihua et al. 2009 was used for extraction and isolation of the essential oils in the Department of Pharmaceutical and Medicinal Chemistry. The fresh dried powdered leaves were completely immersed in distilled water, then hydro-distilled in a full glass Clevenger-type apparatus (India) to give reddish-yellow oil for *P. glandulosus* and very importantly white-clear oil for *C. rigidus*. During heating, generated steam ruptured the cell walls of the leaves and released the oil present in leaves. This process was continued for four hours for maximum oil recovery. The oil was allowed to stand for sufficient time, to be clear, and then it was collected carefully after draining out condensed water. The oil extracted from sample was found containing fraction of water, which was removed by adding small amount of anhydrous sodium sulphate. The essential oil samples were rolled with aluminium foil and stored in the dark at -4℃. The amount of oil obtained from plant materials was calculated as: Oil (% w/w) = Observed mass of oil (g)/Weight of sample (g) × 100 (Aihua et al., 2009).

3.4 Larvicidal and pupicidal bioassays

Larval and pupal bioassays were conducted according to the WHO standard procedure (WHO, 2005) to determine the toxicity of the plant essential oils against *Ae. aegypti, Cx. quinquefasciatus* and *An. gambiae* 4th instar larvae and early pupae. Stock solutions of the essential oils were made using Tween 80 as emulsifier to facilitate the dissolving of material in water. The stock solutions were further diluted up to 10 ml by adding tap water. From these stocks, various

concentrations ranging from 12.5~150 ppm for *P. glandulosus* and from 50~250 ppm for *C. rigidus* against larvae; from 25~300 ppm for both plants against pupae except *C. rigidus* against *Cx. quinquefasciatus* (100~500 ppm) were used. For comparison, a commercial formulation of WARRIOR® 1000 EC (100 % DDVP: 2,2-dichlorovinyl dimethyl phosphate) larvicide and pupicide (2000 ppm, recommended concentration) bought from Awka market (Anambra State, Nigeria) was used as positive control. 1 ml of Tween 80 in 99 ml of tap water was used as negative control. These controls were set up for each replicate, mosquito species and plant. All the concentrations were chosen after a preliminary test for each essential oil and mosquito species. Twenty-five 4th instar mosquito larvae or early pupae were released into each 250 ml beaker containing 100 ml of the aliquot and mortality was recorded after 24 h of exposure. No food was provided either to the tests or controls during the experiments. The dead larvae and pupae were expressed as percentage mortality at each concentration. In cases where bioassay tests showed more than 20% (negative) control mortality, these were discarded and repeated. However when negative control mortality ranged from 5-20%, the observed percentage mortality was corrected by Abbott's formula. The larvae or pupae were considered as dead, if they were not responsive to a gentle prodding with a fine needle. All bioassays were carried out at room temperature of (27±2)℃ and (79±2)% RH. Experiments were set in four replicates along with controls.

% Mortality = (number of larvae or pupae dead / total number of larvae or pupae used) * 100.

Corrected Mortality (%) = [(% test mortality - % control mortality) / (100 − control mortality)] x 100 (Abbott, 1925).

3.5 Statistical analysis

The corrected mortality was determined using Abbott's (1925) formula whenever required. The percentage of mortality data were subjected to ANOVA procedure using Statistical Package for Social Science (SPSS 17.0). Duncan test (P=0.05) was applied for mean separation. Probit analysis (Finney, 1971) was applied to determine lethal dosages causing 50 % (LC_{50}) and 90 % (LC_{90}) mortality of larvae and pupae 24 h post exposure.

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