Hindawi
International Journal of Zoology
Volume 2020, Article ID 4952041, 9 pages
https://doi.org/10.1155/2020/4952041

Research Article

Combined Effect of Methanol Extracts and Essential Oils of *Callistemon rigidus* (Myrtaceae) and *Eucalyptus camaldulensis* (Myrtaceae) against *Anopheles gambiae* Giles larvae (Diptera: Culicidae)

Lame Younoussa,[1,2] **Francine Kenmoe**,[3] **Mallam Kary Oumarou**,[3] **Aurore Christelle Sengue Batti**,[2] **Joseph Lebel Tamesse**,[4] **and Elias Nchiwan Nukenine**[3]

[1]*Institute of Medical Research and Medicinal Plants Studies, P.O. Box 13033, Yaoundé, Cameroon*
[2]*Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde 1, P.O. Box 812, Yaounde, Cameroon*
[3]*Department of Biological Sciences, Faculty of Science, University of Ngaoundéré, P.O. Box 454, Ngaoundere, Cameroon*
[4]*Department of Biological Sciences, Higher Teacher's Training College, University of Yaounde I, P.O. Box 47, Yaounde, Cameroon*

Correspondence should be addressed to Lame Younoussa; younoussalame@yahoo.com

Received 2 October 2019; Accepted 8 February 2020; Published 28 April 2020

Academic Editor: João Pedro Barreiros

Biopesticides from botanicals are nowadays actively encouraged in the mosquito control program because of their low mammalian toxicity, biodegradability, and target specificity. However, leaf methanolic extracts and essential oils of *Callistemon rigidus* and *Eucalyptus camaldulensis* were evaluated individually and in combination on third- and fourth-instar larvae of *Anopheles gambiae*. The extracts were tested individually and in combination at doses of 125, 250, 500, and 1000 ppm while essential oils were applied at 25, 50, 100, and 200 according to the standard protocol of WHO. The commercial insecticide Bi-one tested at the recommended dose of 1000 ppm was used as positive control while the solution of tap water containing 0.5 ml of methanol was used as negative control. The mortality of the larvae was recorded after 24 h postexposure. In the results, 100% mortality of the larvae was recorded with the extracts of *C. rigidus* and the combinations E50% : C50% and E25% : C75% of the plants as well as positive control (1000 ppm). Similarly, essential oils of the two plants and their combinations caused 100% mortality of the larvae. Among the various combinations of the extracts and essential oils, only the combination E75% : C25% of essential oils presented a synergistic effect. Therefore, the essential oil combination (E75% : C25%) of the plants *E. camaldulensis* and *C. rigidus* is recommended in order to promote the its use in the form of natural biocide in the implementation of effective insect controls against the mosquito larvae, vector of malaria.

1. Introduction

In sub-Saharan Africa, malaria still remains one of the most dangerous diseases, responsible for the millions of deaths annually, and children under 5 years old, pregnant women, and persons with HIV/AIDS are the most unwell from that disease [1]. In 2017, approximately 219 million cases were reported with 435,000 deaths recorded from the disease [2]. That deathful disease caused by the *Plasmodium* spp parasites is transmitted to humans through the bites of mosquitoes belonging to the genera of *Anopheles*, and the mosquito species *Anopheles gambiae* Giles is the main vector of malaria in the sub-Saharan African countries. However, current methods put in place to tackle malaria are facing problems of drug side effects expressed in the patients and the development of parasite resistance to drugs currently in use for treatment. Besides, no licensed malaria vaccine exists since numerous malaria vaccine candidates (more than 30 *Plasmodium falciparum* vaccines) targeting either preerythrocytic, blood, or sexual stages of the parasite life cycle are still under clinical trials [3, 4]. For that, vector control remains the best method to

lower the rate of disease transmission in the population. Unfortunately, insect vector control is also facing numerous difficulties because of the misuse of synthetic insecticides in agriculture and insect pest control programmes leading to environmental pollution, causing an ecological imbalance. Specifically, the synthetic insecticides in use are toxic not only to humans but also to nontarget organisms without omitting the problem of the development of insecticide resistance and the resurgence of new pest species [5, 6]. Thus, in recent years around the world, natural products raised the attention of researchers to look for new alternative solutions to reduce the excessive use of these synthetic pesticides. Among these alternatives of which nature presents, botanical-derived products are of particular interest since they are less toxic, biodegradable, and target-specific [7–9]. Plants are rich in bioactive chemical secondary metabolites and have proven their insecticidal activities by killing or repelling insects [10–13].

Native from Australia and belonging to the family of Myrtaceae, *Eucalyptus camaldulensis* is a high tree largely widespread in the word. The essential oil in this plant is rich in oxygenated and nonoxygenated monoterpenes as well as sesquiterpenes [14]. Previously, tannins, flavonoids, glycosides, and sterols were reported in different parts of that plant [15]. Several previous studies reported the mosquitocidal activity of *Eucalyptus camaldulensis* against *Culex pipiens* and *Anopheles stephensi* mosquito species [16–18].

Commonly called stiff bottlebrush, *Callistemon rigidus* (Myrtaceae) is a bushy tree with narrow, pointed, dark leaves and pink-red flowers, found wildly only in Australia and nowadays widespread worldwide. Cineol, flavone, flavonol, and triterpenoid are the major phytocomponents found in the essential oil of *C. rigidus* [19] while tannins and flavonoids are found in the leaves of the plant [20]. The toxic effect of *C. rigidus* essential oils was reported against *Anopheles gambiae*, *Aedes aegypti*, and *Culex quinquefasciatus* mosquito species [21]. The leaf extracts and fractions of the plant were also effective against the bean beetle *Callosobruchus maculatus* [22].

Synergistic effect of the combination of the plant extracts was widely documented in the literature, and plant combination might increase the efficacy instead of each single plant used and consequently may prevent insect resistance issues [23]. Described as any kind of positive interaction between drugs or insecticides, synergism can take place between the constituents of a single extract as well as in a mixture of herbs since herbalists have always insisted that better results are obtained with whole plant extracts and combinations of these rather than with isolated compounds [24]. To search for the improvement of the insecticidal efficacy of plant products and to prevent insecticide resistance problem, this present investigation aimed to assess the synergistic activity of the blend of methanol extracts and essential oils of *Eucalyptus camaldulensis* and *Callistemon rigidus* against the third- and fourth-instar larvae of the main malarial vector *Anopheles gambiae* in the laboratory conditions.

2. Materials and Methods

2.1. Plant Material Collection and Processing. The green leaves of *Callistemon rigidus* and *Eucalyptus camaldulensis* were harvested around the campus of the University of Ngaoundere, Cameroon, in December 2017 and identified by Prof. Pierre-Marie Mapongmetsem, botanist of the Faculty of Science, University of Ngaoundere, Cameroon. The leaves of the two plant species were shade-dried for 10 days at ambient laboratory conditions ($24 \pm 2^{\circ}C$; $76 \pm 4\%$ HR), grounded in the wood mortar, and passed through 0.4 mm mesh size sieve. Each plant powder obtained was stored in the dark bottles at the ambient temperature until their extraction.

2.2. Plant Methanol Extraction. The methanol extract of each plant species was extracted by macerating 250 g of each plant powder in 2500 mL of methanol. The maceration was stirred twice a day and after 72 h and was filtered using Whatman paper No. 1. The methanol in the filtrate was evaporated in open air, and the dried methanol plant extract was stored in the dark glass at $4^{\circ}C$ until its use for bioassay and phytochemical screening. The extraction yield was calculated by the following formula:

$$\text{Methanol extraction yield (\%)} = \frac{\text{weight of the extract obtained}}{\text{weight of plant powder used}} \times 100. \quad (1)$$

2.3. Phytochemical Screening of the Methanol Extract. The methanol extracts of *C. rigidus* and *E. camaldulensis* were submitted to the qualitative phytochemical screening tests to identify some anti-insect phytochemical compounds including alkaloids, flavonoids, saponins, tannins, polyphenols, and terpenoids which, according to the literature, possess an insecticidal property. The methods performed by Harborne [25], Evans and Trease [26], and Prashant et al. [27] were performed to determine these phytocomponents targeted.

2.4. Extraction of Essential Oils. The essential oils of *C. rigidus* and *E. camaldulensis* were extracted by hydrodistillation process using Dean–Stark apparatus. Indeed, 200 g of green leaves of each plant species was mixed with 500 mL of distilled water and submitted to hydrodistillation procedure for 3 h. Floral water and essential oil were separated using separating funnel, and traces of water in the essential oil were completely removed with anhydrous sodium sulfate. Each dried plant essential oil obtained was kept in dark glass and stored at $4^{\circ}C$ until use for the test. The essential oil extraction yield was calculated using the following formula:

$$\text{Essential oil extraction yield (\%)} = \frac{\text{weight of the oil recovered}}{\text{weight of the fresh leaves used}} \times 100. \quad (2)$$

2.5. Mosquito Species. To establish the colony, eggs of the laboratory strain of *Anopheles gambiae* were collected from OCEAC at Yaounde, Cameroon, in February 2018. In the insectarium of the Laboratory of Applied Zoology of the University of Ngaoundere, mosquito eggs were transferred into the bucket containing tap water and the hatched larvae were reared according to the standard protocol of WHO [28]. Larvae were fed with TetraMin® (Tetra GmbH, Germany) and were maintained under ambient condition of the insectarium ($27 \pm 2^{\circ}$C; $74 \pm 4\%$ r.h.). Third and fourth instars of mosquito larvae were used for the experiments.

2.6. Larvicidal Bioassay. The larvicidal efficacy of the methanol extracts and essential oils of *E. camaldulensis* and *C. rigidus* tested singly or in binary combination against *A. gambiae* larvae was evaluated according to the standard protocol described by WHO [29]. The binary combinations of the methanol extracts or essential oils of *E. camaldulensis* with *C. rigidus* in proportions of 25% + 75%, 50% + 50%, and 75% + 25% representing combinations of E25% : C75%, E50% : C50%, and E75% : C25%, respectively, were prepared. Plant methanol extracts and essential oils individually or in binary combination were dissolved in 0.5 mL of Tween-80, and concentrations of 125, 250, 500, 1000, and 2000 ppm for plant methanol extracts and of 25, 50, 100, 200, and 400 ppm for essential oils were prepared in the volume of 100 mL of solution in the plastic cups (250 mL). The negative control consisted to add 0.5 mL to 99.5 mL of tap water while Bi-one™ (1000 ppm) was used as a positive control. A total of 25 third- and fourth-instar larvae were transferred into each solution test preparation, and each dose was repeated 4 times. Larval mortality was recorded after 24 h posttreatment, and larva was declared dead, if it is no longer moving even after pinching with an entomological needle. The larval mortality percentage was calculated and then corrected using Abbott [30] formula if the larval mortality percent in the negative control ranged between 5% and 20% according to the following formula:

$$\begin{aligned}\text{Mortality percent } (\%) &= \frac{\text{number of dead larvae}}{\text{total number of larvae used}} \times 100,\\ \text{Corrected mortality percent } (\%) &= \left[\frac{(\text{NC} - \text{NT})}{\text{NC}}\right] \times 100,\end{aligned} \tag{3}$$

where NC = percentage of dead larvae in the control and NT = percentage of dead larvae in the test.

After the calculation of LC_{50} values of two plants extracts and essential oils tested singly or in combination, cotoxicity index of each combination was determined as follows:

$$\begin{aligned}\text{Toxicity index (TI) of E} &= 100 \text{ and toxicity index (TI) of C}\\ &= \left[\frac{LC_{50} \text{ of E}}{LC_{50} \text{ of C}}\right] \times 100,\\ \text{Observed TI of the mixture} &= \left[\frac{LC_{50} \text{ of E}}{LC_{50} \text{ of the mixture}}\right] \times 100,\\ \text{Observed TI of the mixture} &= \left[\frac{LC_{50} \text{ of E}}{LC_{50} \text{ of the mixture}}\right] \times 100,\\ \text{Theoretical TI of the mixture} &= \text{TI of E} \times \%\text{ of E in the mixture}\\ &\quad + \text{TI of C} \times \%\text{ of C in the mixture},\\ \text{Cotoxicity index} &= \left[\frac{\text{observed TI of the mixture}}{\text{theoretical TI of the mixture}}\right] \times 100,\end{aligned} \tag{4}$$

where E represents extract or essential oil of *E. camaldulensis* and C represents extract or essential oil of *C. rigidus*.

When one component of the mixture (extract or essential oil of *C. rigidus* for example) causes a low mortality (<20%) at all doses tested, cotoxicity index of the combination would be calculated as follows:

$$\text{Cotoxicity index} = \left[\frac{LC_{50} \text{ of E alone}}{LC_{50} \text{ of the mixture}}\right] \times 100. \tag{5}$$

Then, according to Sun and Johnson [31],

(i) If cotoxicity index is less than 80, it is considered as antagonistic action

(ii) If cotoxicity index is between 80 and 120, it is considered as additive action

(iii) If cotoxicity is greater than 120, it is considered as synergistic action

Synergistic factors were also calculated according to Kalyanasundaram and Das [32] method as follows:

$$\begin{aligned}&\text{Synergistic factor (SF)}\\ &= \frac{LC_{50} \text{ of the plant extract or essential oil alone}}{LC_{50} \text{ of the mixture}}.\end{aligned} \tag{6}$$

Value of SF > 1 indicates synergistic action and SF < 1 indicates the antagonistic action.

2.7. Statistical Analyses. Data of the corrected mortality percentage of larvae were submitted to analysis of variance (ANOVA) using SPSS (Statistical Package for the Social Sciences) software version 16.0. Mean separation was

performed using Tukey test ($P = 0.05$). Probit analysis [33] was applied to determine the lethal concentration that caused 50% (LC_{50}) and 95% (LC_{95}) mortality of mosquito larvae.

3. Results

3.1. Extraction Yield. The extraction yield of the leaves of two plant species *C. rigidus* and *E. camaldulensis* is presented in Table 1. When extracted with the methanol solvent, the yield obtained from the leaves of *C. rigidus* (30.71% (w/w)) was slightly high compared to the yield obtained from the leaves of *E. camaldulensis* (23.79% (w/w)). Similarly for essential oils, the yield obtained from the leaves of *C. rigidus* was 1.20% (w/w) which was high compared to the yield obtained from the leaves of *E. camaldulensis* (0.84% (w/w)).

Table 2 presents the result of the phytochemical screening of the methanol extract of *C. rigidus* and *E. camaldulensis*. In the two methanol plant extracts, the six phytochemical constituents targeted including alkaloids, flavonoids, tannins, saponins, terpenoids, and polyphenols were present in variable concentration. In the methanol extract of *C. rigidus*, alkaloids, saponins, and polyphenols were found in high concentration compared to the other constituents present in the plant. Concerning the methanolic extract of *E. camaldulensis*, the plant is highly concentrated in tannins and polyphenols.

The mortality percent of the larvae of *A. gambiae* treated with the methanol extracts of *E. camaldulensis* and *C. rigidus* applied in single each and in binary combination and their LC_{50} as well as LC_{95} (ppm) values 24 h postexposure are presented in Table 3. Applied singly or in combination, the methanol extracts of the two plants exerted a significant ($P < 0.05$) toxicity activity against the mosquito larvae and that efficacy augments with the increase in concentration. Tested singly, a significant larval mortality ranging from 0% at 125 ppm to 98% at 1000 ppm ($F_{(5,18)} = 350.96$; $P < 0.001$) with *E. camaldulensis* extract and from 83% (125 ppm) to 100% (1000 ppm) ($F_{(5,18)} = 1528.66$; $P < 0.001$) with *C. rigidus* extract was recorded. In combination, the mixture E25% : C75% caused significantly high mosquito larval mortality ranging from 57% at 125 ppm to 100% at 1000 ppm ($F_{(5,18)} = 671.17$; $P < 0.001$) compared to the other combinations. Among the two plant extracts, *C. rigidus* methanol extract ($LC_{50} = 39.15$ ppm and $LC_{90} = 319.80$ ppm) was the most potent compared to *E. camaldulensis* extract ($LC_{50} = 408.90$ ppm and $LC_{90} = 849.50$ ppm) when tested on mosquito larvae. However, the combination of the two plant extract lowers the efficacy of *C. rigidus* by increasing the LC_{50} values (117.91, 183.37, and 106.21 ppm for combinations E75% : C25%, E50% : C50%, and E25% : C75%, respectively) compared to LC_{50} of *C. rigidus* tested singly.

The synergistic factor and cotoxicity index of the combination of the essential oils of *E. camaldulensis* and *C. rigidus* against the larvae of *A. gambiae* 24 h posttreatment are presented in Table 4. From these results, only the combination *E. camaldulensis* 75% and *C. rigidus* 25% (synergistic factor = 1.031 and cotoxicity index = 103.177 (between 80 and 120)) showed an additive action against the larvae of *A. gambiae*, 24 h postexposure. The combinations E50% : C50% (synergistic factor = 0.389 and cotoxicity index = 38.969 (less than 80)) and E50% : C50% (synergistic factor = 0.476 and cotoxicity index = 47.632 (less than 80)) were revealed as the bad mixtures since they exhibited each an antagonistic effect against on the mortality of the *A. gambiae* larvae after 24 h posttreatment.

The percentage mortality of *A. gambiae* larvae treated with the essential oils of *E. camaldulensis* and *C. rigidus* applied singly and in binary combination and their LC_{50} as well as LC_{95} (ppm) values 24 h postexposure are presented in Table 5. The essential oil of *E. camaldulensis* tested singly caused 31% larval mortality only at the highest tested dose of 200 ppm while the positive control Bi-one exhibited 100% mortality of *A. gambiae* larvae. The essential oil of *C. rigidus* tested also singly caused a significant ($F_{(5,18)} = 376.93$, $P < 0.001$) larval mortality ranging from 0% at 25 ppm to 88% at 200 ppm. In binary combination, the mixture E75% : C25% caused a significant ($F_{(5,18)} = 389.02$; $P < 0.001$) larvicidal activity ranging from 0% (25 ppm) to 89% (200 ppm) while the combination E50% : C50% exhibited a significant ($F_{(5,18)} = 404.69$; $P < 0.001$) mosquito larval mortality ranging from 0% at 25 ppm to 43% at 200 ppm 24 h postexposure. The combination showed also a significant ($F_{(5,18)} = 691.00$; $P < 0.001$) *A. gambiae* larval mortality ranged from 0% at 25 ppm to 66% at 200 ppm after 24 h posttreatment. In single plant essential oil treatment each, *C. rigidus* essential oil ($LC_{50} = 99.66$ ppm) was revealed to be the most effective compared to *E. camaldulensis* essential oil ($LC_{50} = 223.03$ ppm) against the larvae of *A. gambiae* 24 h postexposure. In binary combination, the mixture E75% : C25% with $LC_{50} = 62.87$ ppm was the best combination compared to E50% : C50% ($LC_{50} = 132.58$ ppm) and E25% : C75% ($LC_{50} = 115.16$ ppm) combination against larvae of *A. gambiae*, 24 h posttreatment.

Table 6 presents the synergistic factor and cotoxicity index of the combination of the essential oils of *E. camaldulensis* and *C. rigidus* against the larvae of *A. gambiae*, 24 h posttreatment. The combination 75% of *E. camaldulensis* essential oil with 25% of *C. rigidus* essential oil (E75% : C25%) with synergistic factor of 2.709 (>2) and the cotoxicity index of 270.908 (>120) significantly optimized the efficacy of the combination generating a synergistic efficacy against mosquito larvae assayed. The combinations *E. camaldulensis* 50% and *C. rigidus* 50% (synergistic factor = 1.039 and cotoxicity index = 103.908) as well as *E. camaldulensis* 25% and *C. rigidus* 75% (synergistic factor = 1.004 and cotoxicity index = 100.428) exhibited an additive action against the larvae of *A. gambiae* 24 h posttreatment.

4. Discussion

In the insect pest control agent research, the insecticide combination approach is encouraged not only to optimize the efficacy of the insecticide products but also to solve the problem of insect resistance and might apparently preserve efficacy for the insecticide product for many years. Only one action among additive, synergistic, or antagonistic effect is expected in the combination of drug or insecticide [34], and

Table 1: Methanol extract and essential oil extraction yields of *C. rigidus* and *E. camaldulensis*.

Plant products	Plant species	Plant material weight used (g)	Yield (%)
Methanol extract	*C. rigidus*	250	30.71
	E. camaldulensis	250	23.79
Essential oils	*C. rigidus*	200	1.20
	E. camaldulensis	200	0.85

Table 2: Phytochemical components of the methanol extracts of *C. rigidus* and *E. camaldulensis*.

Extracts	Alkaloids	Flavonoids	Tannins	Saponins	Terpenoids	Polyphenols
C. rigidus	+++	++	+	+++	+	+++
E. camaldulensis	+	++	+++	+	+	+++

+ = present at low concentration, ++ = present at moderate concentration, and +++ = present at high concentration.

Table 3: Mortality percent of *A. gambiae* larvae treated with the combination of methanol extracts of *E. camaldulensis* and *C. rigidus* and LC_{50} as well as LC_{95} (ppm) values 24 h postexposure.

Combinations	Conc (ppm)	% mortality	Slope ± SE	LC_{50} (LFL-UFL) (ppm)	LC_{95} (LFL-UFL) (ppm)	χ^2
E100%	0	0.00 ± 0.00D	5.18 ± 0.22	408.90 (384.79–434.53)	849.50 (769.31–959.86)	34.93^{**}
	125	0.00 ± 0.00D				
	250	15.00 ± 3.00C				
	500	66.00 ± 5.29B				
	1000	98.00 ± 1.15A				
	Bi-one	100 ± 0.00A				
	$F_{(5,18)}$	350.96^{***}				
E75% : C25%	0	0.00 ± 0.00D	2.93 ± 0.17	117.91 (81.28–147.90)	426.97 (329.72–673.50)	133.33^{***}
	125	47.00 ± 2.51C				
	250	91.00 ± 2.51B				
	500	97.00 ± 1.91AB				
	1000	98.00 ± 1.15AB				
	Bi-one	100 ± 0.00A				
	$F_{(5,18)}$	560.70^{***}				
E50% : C50%	0	0.00 ± 0.00D	4.94 ± 0.23	183.37 (167.85–199.15)	394.28 (346.73–469.01)	57.66^{***}
	125	17.00 ± 1.91C				
	250	92.00 ± 2.58B				
	500	96.00 ± 1.63A				
	1000	100 ± 0.00A				
	Bi-one	100 ± 0.00A				
	F	943.83^{***}				
E25% : C75%	0	0.00 ± 0.00D	3.46 ± 0.24	106.21 (82.49–124.98)	317.17 (265.39–422.31)	65.63^{***}
	125	58.00 ± 2.58C				
	250	93.00 ± 1.91B				
	500	98.00 ± 2.00AB				
	1000	100 ± 0.00A				
	Bi-one	100 ± 0.00A				
	$F_{(5,18)}$	671.17^{***}				
C100%	0	0.00 ± 0.00D	1.80 ± 0.18	39.15 (24.95–53.24)	319.80 (278.04–380.77)	16.67^{ns}
	125	83.00 ± 1.91C				
	250	91.00 ± 1.00B				
	500	98.00 ± 1.15A				
	1000	100 ± 0.00A				
	Bi-one	100 ± 0.00A				
	$F_{(5,18)}$	1528.66^{***}				

Mean of mortality ± standard deviation within a column followed by the same letter did not differ significantly according to Tukey test ($P = 0.05$); $^{ns}P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; LC = lethal concentration; LFL: lower fiducial limit; UFL: upper fiducial limit; Bi-one = positive control tested at 1000 ppm; number of replicates: 4. E100%, 75%, 50%, 25%, and 0% = *E. camaldulensis* 100%, 75%, 50%, 25%, and 0%, respectively; C100%, 75%, 50%, 25%, and 0% = *C. rigidus* 100%, 75%, 50%, 25%, and 0%, respectively.

Table 4: Synergistic factor and cotoxicity index of the combination of the methanol extracts of *E. camaldulensis* and *C. rigidus*.

Combinations	LC_{50} (ppm)	Synergistic factor	Cotoxicity index	Type of action
E100% : C0%	408.90	—	—	—
E75% : C25%	117.91	1.031	103.177	Additive
E50% : C50%	183.37	0.389	38.969	Antagonistic
E25% : C75%	106.21	0.476	47.632	Antagonistic
E0% : C100%	39.15	—	—	—

E100%, 75%, 50%, 25%, and 0% = *E. camaldulensis* 100%, 75%, 50%, 25%, and 0%, respectively; C100%, 75%, 50%, 25%, and 0% = *C. rigidus* 100%, 75%, 50%, 25%, and 0%, respectively.

Table 5: Mortality percent of *A. gambiae* larvae treated with the combination of the essential oils of *E. camaldulensis* and *C. rigidus* and LC_{50} as well as LC_{95} (ppm) values 24 h postexposure.

Combinations	Conc (ppm)	% mortality	Slope ± SE	LC_{50} (LFL-UFL) (ppm)	LC_{95} (LFL-UFL) (ppm)	χ^2
E100%	0	0.00 ± 0.00C	7.35 ± 0.40	223.03 (213.51–233.14)	337.17 (345.86–410.84)	24.54^{ns}
	25	0.00 ± 0.00C				
	50	0.00 ± 0.00C				
	100	0.00 ± 0.00C				
	200	31.00 ± 3.00B				
	Bi-one	100.0 ± 0.00A				
	$F_{(5,18)}$	1729^{***}				
E75% : C25%	0	0.00 ± 0.00D	3.40 ± 0.13	62.87 (52.48–74.23)	191.03 (149.12–278.02)	165.60^{***}
	25	0.00 ± 0.00D				
	50	50.00 ± 4.16C				
	100	81.00 ± 2.51B				
	200	89.00 ± 3.41B				
	Bi-one	100.0 ± 0.00A				
	$F_{(5,18)}$	389.02^{***}				
E50% : C50%	0	0.00 ± 0.00D	2.42 ± 0.09	132.58 (102.77–176.29)	632.22 (397.71–1460.60)	256.36^{***}
	25	0.00 ± 0.00D				
	50	29.00 ± 4.43C				
	100	38.00 ± 2.58BC				
	200	43.00 ± 1.91B				
	Bi-one	100.0 ± 0.00A				
	$F_{(5,18)}$	404.65^{***}				
E25% : C75%	0	0.00 ± 0.00E	3.07 ± 0.11	115.16 (101.71–130.69)	394.96 (318.00–528.64)	84.48^{***}
	25	0.00 ± 0.00E				
	50	20.00 ± 1.63D				
	100	43.00 ± 3.41C				
	200	66.00 ± 2.58B				
	Bi-one	100.0 ± 0.00A				
	$F_{(5,18)}$	691.00^{***}				
C100%	0	0.00 ± 0.00E	3.73 ± 0.14	99.66 (89.32–111.32)	274.76 (229.71–348.72)	81.32^{***}
	25	0.00 ± 0.00E				
	50	21.00 ± 3.41D				
	100	40.00 ± 3.65C				
	200	88.00 ± 3.65B				
	Bi-one	100.0 ± 0.00A				
	$F_{(5,18)}$	376.93^{***}				

Mean of mortality ± standard deviation within a column followed by the same letter did not differ significantly according to Tukey test ($P = 0.05$); $^{ns}P > 0.05$; $^{***}P < 0.001$; LC: lethal concentration; LFL: lower fiducial limit; UFL: upper fiducial limit; Bi-one = positive control tested at 1000 ppm; number of replicates: 4. E100%, 75%, 50%, 25%, and 0% = *E. camaldulensis* 100%, 75%, 50%, 25%, and 0%, respectively; C100%, 75%, 50%, 25%, and 0% = *C. rigidus* 100%, 75%, 50%, 25%, and 0%, respectively.

the aim of any plant combination assay carried out is to obtain synergistic action of the mixture. Results from the larvicidal activity showed that extracts or essential oils of *E. camaldulensis* and *C. rigidus* used singly or in binary combination caused a significant mortality of *A. gambiae* larvae. In the present study, methanol extract of *C. rigidus* tested singly was the most potent against mosquito larvae and its combination with *E. camaldulensis* extract exhibited antagonistic effects. Nevertheless, previous studies showed synergistic action of plant extracts when blended. Thus, the binary combination of ethanol leaf extracts of *Dracaena arborea* and *Vitex doniana* exerted synergistic effects on *Anopheles* mosquito species [35]. Yankanchi et al. [36] tested individually and in combination with *Pongamia glabra* seed extract, three leaf plants extracts

Table 6: Synergistic factor and cotoxicity index of the combination of the essential oils of *E. camaldulensis* and *C. rigidus*.

Combinations	LC_{50} (ppm)	Synergistic factor	Cotoxicity index	Type of action
E100% : C0%	223.03	—	—	—
E75% : C25%	62.87	2.709	270.908	Synergistic
E50% : C50%	132.58	1.039	103.908	Additive
E25% : C75%	115.16	1.004	100.428	Additive
E0% : C100%	99.66	—	—	—

E100%, 75%, 50%, 25%, and 0% = *E. camaldulensis* 100%, 75%, 50%, 25%, and 0%, respectively; C100%, 75%, 50%, 25%, and 0% = *C. rigidus* 100%, 75%, 50%, 25%, and 0%, respectively.

Vitex negundo, Clerodendrum inerme, and *Gliricidia sepium*against fourth-instar larvae of *Aedes aegypti* and found that, tested individually, *C. inerme* (LC_{50} = 292.36 ppm) was the most toxic while the maximum synergistic activity was found in the combination extracts of *C. inerme* 50% with *P. glabra* 50% (LC_{50} = 195.02 ppm) as well as in 50% *V. negundo* with 50% *P. glabra* (LC_{50} = 191.73 ppm). Synergistic efficacy of the mixtures of *Callitris glaucophylla* extracts and *Khaya senegalensis* extracts at 1 : 1 ratio against the fourth earlier instar larvae of *A. aegypti* and *C. annulirostris* within the first 24 h [37]. The efficacy of the two plant extracts against mosquito larvae may be due to their richness in alkaloids, flavonoids, tannins, saponins, terpenoids, and polyphenols which have been previously reported to possess insecticidal properties [37]. These toxic phytocompound substances are ingested orally or through cuticle route which might affect insect physiology balance causing death [38].

Mixing *E. camaldulensis* with *C. rigidus* essential oils in 3 : 1 ratio showed a synergistic activity when tested against *A. gambiae* larvae in the present investigation. This result corroborates with the findings of Ríos et al. [39] in which the essential oils of *Thymus vulgaris* tested individually and the combination of *Lippia origanoides* with *Swinglea glutinosa* exhibited the highest larvicidal activity on *A. aegypti* larvae. Similarly, results showed that the combinations of essential oils extracted from resin of *Aucoumea klaineana, Canarium schweinfurthii*, and *Dacryodes edulis* led to the enhancement of their efficacy and exhibited a significant larvicidal activity against *A. gambiae* [40]. The binary combinations in 1 : 1 ratio of *Syzygium aromaticum* + *Illicium verum*, *S. aromaticum* + *Trachyspermum ammi*, and *I. verum* + *T. ammi* essential oils showed synergistic interactions among the binary mixtures [41]. Zibaee et al. [42] obtained the best repellent activity with the cream formulation containing a combination blend of Rosemary and Chamomile oils against the *A. stephensi* and *C. pipiens* adults. The repellent efficacy of *Azadirachta indica* oil against *A. aegypti* was reinforced when sweet basil oil and lemon eucalyptus oil were added to it [43]. Applied in a ratio of 1 : 4, the combination of the two monoterpenoids thymol and carvacrol enhanced the efficacy of the mixture leading to the significant synergistic action against larvae of *C. pipiens*, compared to the efficacy of single compounds tested individually [44]. A study conducted by Pavela et al. [45] revealed the high larvicidal toxicity of the combination mixtures of carvacrol with carvone, carvacrol with 4-allyanisole, and carvacrol with terpineol, when tested against *C. quinquefasciatus* larvae. Conversely, when combined, garlic and asafoetida essential oils showed antagonistic action compared to their application individually against larvae of *C. pipiens* and *C. restuans* [46]. Similar observation was also reported that essential oil of *Eucalyptus citriodora* used alone showed the best larvicidal activity compared to its combination with *Cymbopogon nardus* oil against *A. gambiae* larvae [47]. The toxicity of the plant extract or essential oil combination mixtures might be due the mixture of the phytocomponents found in the mixture and may be acting in synergy as neurotoxic insecticides interfering in the ligand-gated chloride channel of the mosquito larval nervous system or by blocking octopamine or cholinergic receptors, the important target sites of insect pest control [48–50].

5. Conclusion

The methanol extracts and essential oils of the two plants tested singly or in binary combinations caused a significant larvicidal activity against *A. gambiae* larvae. Tested individually, the methanol extract and essential oil of *C. rigidus* was revealed to be the most potent compared to *E. camaldulensis* against mosquito larvae. However, binary combination of the plant methanol extract exhibited antagonistic action while *E. camaldulensis* (75%) and *C. rigidus* (25%) essential oil blend displayed synergistic effect when applied on mosquito larvae. This makes it a more suitable candidate for the development of new potential eco-friendly larvicide for *A. gambiae* mosquito control in the larvae breeding sites.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

L. Younoussa, J. L. Tamesse, and E. N. Nukenine designed the study. L. Younoussa, M. K. Oumarou, A. C. S. Batti, and F. Kenmoe conducted the experiments. L. Younoussa analyzed the data and wrote the first draft of the manuscript. All authors read, corrected, and approved the final version of the manuscript.

Acknowledgments

The authors are grateful to Prof. Pierre-Marie Mapongmetsem for the identification of plant species. They acknowledge the Laboratory of Chemistry, Faculty of Science, University of Ngaoundere, Cameroon, for plant extractions and phytochemical screening and are also grateful to the Organization of Coordination for the Fight against Endemic Diseases in Central Africa (OCEAC) for providing mosquito eggs used in the present work.

References

[1] V. L. Kiggundu, W. P. O'Meara, R. Musoke et al., "High prevalence of malaria parasitemia and anemia among hospitalized children in Rakai, Uganda," *PLoS One*, vol. 8, no. 12, Article ID e82455, 2013.

[2] WHO, *World Malaria Report*, World Health Organization, Geneva, Switzerland, 2018.

[3] L. Schwartz, G. V. Brown, B. Genton, and V. S. Moorthy, "A review of malaria vaccine clinical projects based on the WHO rainbow table," *Malaria Journal*, vol. 11, no. 1, p. 1, 2012.

[4] WHO, *Tables of Malaria Vaccine Projects Globally, The Rainbow Tables*, 2015, http://www.who.int/immunization/research/development/Rainbow_tables/en/.

[5] G. J. Devine and M. J. Furlong, "Insecticide use: contexts and ecological consequences," *Agriculture and Human Values*, vol. 24, no. 3, pp. 281–306, 2007.

[6] M. Sarwar, N. Ahmad, and M. Toufiq, "Host plant resistance relationshiphs in chickpea (*Cicer arietinum* L.) against gram pod borer (*Helicoverpa armigera* Hubner)," *Pakistan Journal of Botany*, vol. 41, no. 6, pp. 3047–3052, 2009.

[7] V. K. Dua, A. C. Pandey, and A. P. Dash, "Adulticidal activity of essential oil of *Lantana camara* leaves against mosquitoes," *Indian Journal of Medical Research*, vol. 131, pp. 434–439, 2010.

[8] M. Govindarajan, "Mosquito larvicidal and ovicidal activity of *Cardiospermum halicacabum* Linn. (Family: Sapindaceae) leaf extract against *Culex quinquefasciatus* (say.) and *Aedes aegypti* (Linn.) (Diptera: Culicidae)," *European Review for Medical and Pharmacological Science*, vol. 15, no. 7, pp. 787–794, 2011.

[9] J. Subramaniam, K. Kovendan, P. Mahesh Kumar, K. Murugan, and W. Walton, "Mosquito larvicidal activity of Aloe vera (Family: Liliaceae) leaf extract and *Bacillus sphaericus*, against Chikungunya vector, *Aedes aegypti*," *Saudi Journal of Biological Sciences*, vol. 19, no. 4, pp. 503–509, 2012.

[10] K. Sukumar, M. J. Perich, and L. R. Boobar, "Botanical derivatives in mosquito control: a review," *Journal of American Mosquito Control Association*, vol. 72, pp. 210–237, 1991.

[11] F. Candan, M. Unlu, B. Tepe et al., "Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae)," *Journal of Ethnopharmacology*, vol. 87, no. 2-3, pp. 215–220, 2003.

[12] C. C. Joshep, M. M. Ndoils, R. C. Malima, and N. H. M. Nkuniya, "Larvicidal and mosquitocidal extracts, a coumrin, isofavonoids and petrocarpans from *Neorautanenia mitis*," *Transactions for the Royal Society of Tropical Medicine and Hygiene*, vol. 98, pp. 451–455, 2004.

[13] A. Ghosh, N. Chowdhury, and G. Chandra, "Plant extracts as potential mosquito larvicides," *Indian Journal of Medical Research*, vol. 135, pp. 581–598, 2012.

[14] A. Farah, B. Satrani, M. Fechtal, A. Chaouche, and M. Talbie, "Chemical composition, antibacterial and antifungal activities of essential oils from the leaves of *Eucalyptus camaldulensis* and its natural hybrid (clone 583)," *Acta Botanica Gallica (France)*, vol. 148, no. 3, pp. 183–190, 2011.

[15] J. M. Watt and M. G. Breyer-Brandwijk, *The Medicinal and Poisonous Plants of Southern and Eastern Africa*, E.&S. Livingstone, Ltd., London, UK, 2nd edition, 1962.

[16] K. Watanabe, Y. Shono, A. Kakimizu et al., "New mosquito repellent from *Eucalyptus camaldulensis*," *Journal of Agricultural and Food Chemistry*, vol. 41, no. 11, pp. 2164–2166, 1993.

[17] S. M. Nishimura, S. Reza, K. Mahnaz et al., "Phytochemistry and larvicidal activity of *Eucalyptus camaldulensis* against malaria vector, *Anopheles stephensi*," *Asian Pacific Journal of Tropical Medicine*, vol. 3, no. 11, pp. 841–845, 2010.

[18] A. Alouani, T. Ababsia, I. Rahal, N. Rehimi, and H. Boudjelida, "Activity evaluation of botanical essential oils against immature mosquitoes of *Culex pipiens* (Diptera: Culicidae)," *Journal of Entomology and Zoology Studies*, vol. 5, no. 4, pp. 829–834, 2007.

[19] L. Jirovetz, W. Fleischacker, G. Buchbauer, and M. B. Ngassoum, "Analysis of the essential oils of *Callistemon rigidus* (Myrtaceae) from cameroun by GC/FID and GC/MS," *Scientia Pharmaceutica*, vol. 65, pp. 315–319, 1997.

[20] K. G. Praveen, J. Renuka, J. Shweta, and S. Archana, "A review on biological and phytochemical investigation of plant genus *Callistimon*," *Asian Pacifique Journal of Tropical Biomedicine*, pp. S1906–S1909, 2012.

[21] Y. S. P. Danga, C. O. Osimone, L. Younoussa, and E. N. Nukenine, "Larvicidal and pupicidal activities of *Plectranthus glandulosus* and *Callistemon rigidus* leaf essential oils against three mosquito species," *Journal of Mosquito Research*, vol. 4, no. 2, pp. 5–14, 2014.

[22] S. P. Y. Danga, E. N. Nukenine, L. Younoussa, C. Adler, and C. O. Esimone, "Efficacy of *Plectranthus glandulosus* (Lamiaceae) and *Callistemon rigidus* (Myrtaceae) leaf extract fractions to *Callosobruchus maculatus* (Coleoptera: Bruchidae)," *Journal of Insect Science*, vol. 15, no. 1, pp. 139–145, 2015.

[23] M. B. Isman, "Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world," *Annual Review of Entomology*, vol. 51, no. 1, pp. 45–66, 2006.

[24] E. M. Williamson, "Synergy and other interactions in phytomedicines," in *Trease and Evans Pharmacognosy*, pp. 53–61, Elsevier, Amsterdam, Netherlands, 16th edition, 2009.

[25] J. B. Harborne, *Phytochemical Methods. A Guide to Modern Techniques of Plant Analysis*, Chapman & Hall, London, UK, 1973.

[26] G. E. Trease and W. C. Evans, *Pharmacognosy*, Macmillian Publishers Naturalist, London, UK, 1978.

[27] T. Prashant, K. Bimlesh, K. Mandeep, K. Gurpreet, and K. Harleen, "Phytochemical screening and extraction: a review," *Internationale Pharmaceutica Sciencia*, vol. 1, no. 1, pp. 98–106, 2011.

[28] WHO, "Report of the World Health Organization," *informal consultation on the evaluation and testing of insecticides*, World Health Organization, Geneva, Switzerland, 1996.

[29] WHO, *Guidelines for Laboratory and Field Testing of Mosquito Larvicides*, World Health Organization, Geneva, Switzerland, 2005.

[30] W. S. Abbott, "A method of computing the effectiveness of an insecticide," *Journal of Economic Entomology*, vol. 18, no. 2, pp. 265–267, 1925.

[31] Y.-P. Sun and E. R. Johnson, "Synergistic and antagonistic actions of insecticide-synergist combinations and their mode of action," *Journal of Agricultural and Food Chemistry*, vol. 8, no. 4, pp. 261–266, 1960.

[32] M. Kalyanasundaram and P. K. Das, "Larvicidal and synergistic activity of plant extracts for mosquito control," *Indian Journal of Medical Research*, vol. 82, pp. 19–23, 1985.

[33] D. J. Finney, *Probit Analysis*, Cambridge University Press, London, UK, 1971.

[34] A. C. Nelson and T. A. Kursar, "Interactions among plant defense compounds: a method for analysis," *Chemoecology*, vol. 9, no. 2, pp. 81–92, 1999.

[35] C. V. Nnamani, H. O. Oselebe, and A. Ogbonna, "Effect of leaf extracts of *Draceana aborea* L. and *Vitex doniana* sweet on the larvae of *Anopheles* mosquito," *Animal Research International*, vol. 5, no. 2, pp. 835–837, 2008.

[36] S. R. Yankanchi, O. V. Yadav, and G. S. Jadhav, "Synergistic and individual efficacy of certain plant extracts against dengue vector mosquito, *Aedes aegypti*," *Journal of Biopesticides*, vol. 7, no. 1, pp. 22–28, 2014.

[37] E. A. Shaalan, D. V. Canyon, M. W. F. Younes, H. Abdel-Wahab, and A. H. Mansour, "Synergistic efficacy of botanical blends with and without synthetic insecticides against *Aedes aegypti* and *Culex annulirostris* mosquitoes," *Journal of Vector Ecology*, vol. 30, no. 2, pp. 284–288, 2005.

[38] R. S. Rattan, "Mechanism of action of insecticidal secondary metabolites of plant origin," *Crop Protection*, vol. 29, no. 9, pp. 913–920, 2010.

[39] N. Ríos, E. E. Stashenko, and J. E. Duque, "Evaluation of the insecticidal activity of essential oils and their mixtures against *Aedes aegypti* (Diptera: Culicidae)," *Revista Brasileira de Entomologia*, vol. 61, no. 4, pp. 307–311, 2017.

[40] L. C. Obame, J. P. Ondo, G. S. Padzys et al., "Ovicidal and larvicidal activities against *Anopheles gambiae*, antioxidant and antibacterial proprieties of *Aucoumea klaineana* Pierre, *Canarium schweinfurthii* Engl and *Dacryodes edulis* (G. Don) Lam essential oils from Gabon," *International Journal of Pharmacology Research*, vol. 6, no. 1, pp. 68–75, 2016.

[41] G. N. Pandiyan, N. Mathew, and S. Munusamy, "Larvicidal activity of selected essential oil in synergized combinations against *Aedes aegypti*," *Ecotoxicology and Environmental Safety*, vol. 174, pp. 549–556, 2019.

[42] I. Zibaee, H. M. Farahani, J. M. Razi, and M. N. Moghaddam, "Synergistic effect of two medicinal plants in cream formulation on repellency of vector mosquito and rove beetle," *Journal of Entomology and Zoology Studies*, vol. 4, no. 4, pp. 73–79, 2016.

[43] K. P. Kiplang'at and R. W. Mwangi, "Synergistic repellent activity of plant essential oils against *Aedes aegypti* on rabbit Skin," *International Journal of Mosquito Research*, vol. 1, no. 4, pp. 55–59, 2014.

[44] M. R. Youssefi, M. A. Tabari, A. Esfandiari et al., "Efficacy of two monoterpenoids, carvacrol and thymol, and their combinations against eggs and larvae of the west nile vector *Culex pipiens*," *Molecules*, vol. 24, no. 10, p. 1867, 2019.

[45] R. Pavela, F. Maggi, R. Iannarelli, and G. Benelli, "Plant extracts for developing mosquito larvicides: from laboratory to the field, with insights on the modes of action," *Acta Tropica*, vol. 193, pp. 236–271, 2019.

[46] E. J. Mutiri, J. L. Ramirez, B. Zilkowski, L. B. Flor-Weiler, and A. P. Rooney, "Ovicidal and larvicidal effects of garlic and asafoetida essential oils against west nile virus vectors," *Journal of Insect Science*, vol. 18, no. 2, pp. 1–6, 2018.

[47] L. C. Obame-Engonga, C. Sima-Obiang, R. L. Ngoua-Meye-Misso et al., "Larvicidal and ovicidal properties against *Anopheles gambiae*, antioxidant and antibacterial activities of the combination of essential oils *Eucalyptus citriodora, Cymbopogon giganteus* and *Cymbopogon nardus* from Gabon," *Journal of Multidisciplinary Engineering Science and Technology*, vol. 4, no. 8, pp. 7887–7894, 2017.

[48] C. M. Priestley, E. M. Williamson, K. A. Wafford, and D. B. Sattelle, "Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human GABAA receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*," *British Journal of Pharmacology*, vol. 140, no. 8, pp. 1363–1372, 2003.

[49] F. Tong and J. R. Coats, "Effects of monoterpenoid insecticides on [^{3}H]-TBOB binding in house fly GABA receptor and $^{36}Cl^-$ uptake in American cockroach ventral nerve cord," *Pesticide Biochemistry and Physiology*, vol. 98, no. 3, pp. 317–324, 2010.

[50] V. López, M. Cascella, G. Benelli, F. Maggi, and C. Gómez-Rincón, "Green drugs in the fight against Anisakis simplex-larvicidal activity and acetylcholinesterase inhibition of *Origanum compactum* essential oil," *Parasitology Research*, vol. 117, no. 3, pp. 861–867, 2018.