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)

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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 world. 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 ± 2°C, 76 ± 4% HR), ground 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°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°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 ± 2°C; 74 ± 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 0.5mL 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 100mL of solution in the plastic cups (250mL). The negative control consisted to add 0.5mL to 99.5mL 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 24h 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:

\[
\text{Corrected mortality percent} = \left( \frac{\text{NC} - \text{NT}}{\text{NC}} \right) \times 100, 
\]

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

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

\[
\text{Toxicity index (TI)} \text{ of E} = 100 \text{ and toxicity index (TI)} \text{ of C} = \left( \frac{\text{LC}_{50} \text{ of E}}{\text{LC}_{50} \text{ of C}} \right) \times 100, 
\]

\[
\text{Observed TI of the mixture} = \left( \frac{\text{LC}_{50} \text{ of E}}{\text{LC}_{50} \text{ of the mixture}} \right) \times 100, 
\]

\[
\text{Theoretical TI of the mixture} = \text{TI of E} \times \% \text{ of E in the mixture} + \text{TI of C} \times \% \text{ of C in the mixture}, 
\]

\[
\text{Cotoxicity index} = \left( \frac{\text{observed TI of the mixture}}{\text{theoretical TI of the mixture}} \right) \times 100, 
\]

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:

\[
\text{Cotoxicity index} = \left( \frac{\text{LC}_{50} \text{ of E alone}}{\text{LC}_{50} \text{ of the mixture}} \right) \times 100. 
\]

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:

\[
\text{Synergistic factor (SF)} = \left( \frac{\text{LC}_{50} \text{ of the plant extract or essential oil alone}}{\text{LC}_{50} \text{ of the mixture}} \right). 
\]

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 ($F(3,18) = 376.93; P < 0.001$) larval mortality ranging from 0% at 25 ppm to 88% at 200 ppm. In binary combination, the mixture E75%:C25% caused a significant ($F(3,18) = 389.02; P < 0.001$) larvicidal activity ranging from 0% (25 ppm) to 89% (200 ppm) while the combination E50%:C50% exhibited a significant ($F(3,18) = 404.69; P < 0.001$) mosquito larval mortality ranging from 0% at 25 ppm to 43% at 200 ppm 24 h postexposure. The combination showed also a significant ($F(3,18) = 691.00; P < 0.001$) *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<sub>50</sub> as well as LC<sub>95</sub> (ppm) values 24h postexposure. |
|-----------------------------------------------|
| **Combinations** | **Conc (ppm)** | **% mortality** | **Slope ± SE** | **LC<sub>50</sub> (LFL-UFL) (ppm)** | **LC<sub>95</sub> (LFL-UFL) (ppm)** | **χ<sup>2</sup>** |
|---------------------|----------------|----------------|---------------|-----------------------------------|-----------------------------------|------|
| 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         | 943.83***      |               |                                   |                                   |      |
|                     | F(5,18)        |               |               |                                   |                                   |      |
| 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         | 671.17***      |               |                                   |                                   |      |
|                     | F(5,18)        |               |               |                                   |                                   |      |
| C100%               | 0              | 0.00 ± 0.00D   | 1.80 ± 0.18   | 39.15 (24.95–53.24)             | 319.80 (278.04–380.77)            | 16.67ns |
|                     | 125            | 83.00 ± 1.91C  |               |                                   |                                   |      |
|                     | 250            | 91.00 ± 1.00B  |               |                                   |                                   |      |
|                     | 500            | 98.00 ± 1.15A  |               |                                   |                                   |      |
|                     | 1000           | 100 ± 0.00A    |               |                                   |                                   |      |
|                     | Bi-one         | 1528.66***     |               |                                   |                                   |      |

Mean of mortality ± standard deviation within a column followed by the same letter did not differ significantly according to Tukey test (\(P = 0.05\)); **\(P > 0.05\); *\(P < 0.01\); **\(P < 0.001\); 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.
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 4: Synergistic factor and cotoxicity index of the combination of the methanol extracts of *E. camaldulensis* and *C. rigidus.*

| Combinations     | LC50 (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      | —                  | —                | —             |

### Table 5: Mortality percent of *A. gambiae* larvae treated with the combination of the essential oils of *E. camaldulensis* and *C. rigidus* and LC50 as well as LC95 (ppm) values 24 h postexposure.

| Combinations     | Conc (ppm) | % mortality | Slope ± SE | LC50 (LFL-UFL) (ppm) | LC95 (LFL-UFL) (ppm) | \( \chi^2 \)  |
|------------------|------------|-------------|------------|----------------------|----------------------|---------------|
|                  | 0          | 0.00 ± 0.00C| 7.35 ± 0.40| 223.03 (213.51–233.14)| 337.17 (345.86–410.84)| 24.54**       |
| E100%            | 25         | 0.00 ± 0.00C| 3.40 ± 0.13| 62.87 (52.48–74.23)  | 191.03 (149.12–278.02)| 165.60***     |
|                  | 50         | 0.00 ± 0.00C| 2.42 ± 0.09| 132.58 (102.77–176.29)| 632.22 (397.71–1460.60)| 256.36***     |
|                  | 100        | 0.00 ± 0.00C| 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
|                  | 200        | 31.00 ± 3.00B| —            | —                    | —                    | —             |
| Bi-one           |            | 100.0 ± 0.00A| 1729**       | —                    | —                    | —             |
|                  | 0          | 0.00 ± 0.00D | 3.40 ± 0.13| 62.87 (52.48–74.23)  | 191.03 (149.12–278.02)| 165.60***     |
| E75% : C25%      | 25         | 0.00 ± 0.00C| 2.42 ± 0.09| 132.58 (102.77–176.29)| 632.22 (397.71–1460.60)| 256.36***     |
|                  | 50         | 50.00 ± 4.16C| 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
|                  | 100        | 43.00 ± 1.91B| —            | —                    | —                    | —             |
|                  | 200        | 100.0 ± 0.00A| 404.65**     | —                    | —                    | —             |
| Bi-one           |            | 100.0 ± 0.00A| 389.02**     | —                    | —                    | —             |
|                  | 0          | 0.00 ± 0.00E | 2.42 ± 0.09| 132.58 (102.77–176.29)| 632.22 (397.71–1460.60)| 256.36***     |
| E50% : C50%      | 25         | 0.00 ± 0.00E | 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
|                  | 50         | 29.00 ± 4.43C| 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
|                  | 100        | 43.00 ± 3.41C| 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
|                  | 200        | 66.00 ± 2.58B| 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
| Bi-one           |            | 100.0 ± 0.00A| 461.00***    | —                    | —                    | —             |
|                  | 0          | 0.00 ± 0.00E | 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
| E25% : C75%      | 25         | 0.00 ± 0.00E | 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
|                  | 50         | 21.00 ± 3.41D| 3.07 ± 0.11| 115.16 (101.71–130.69)| 394.96 (318.00–528.64)| 256.36***     |
|                  | 100        | 40.00 ± 3.65C| 3.07 ± 0.11| 99.66 (89.32–111.32) | 274.76 (229.71–348.72)| 81.32***      |
|                  | 200        | 88.00 ± 3.65B| 3.07 ± 0.11| 99.66 (89.32–111.32) | 274.76 (229.71–348.72)| 81.32***      |
| Bi-one           |            | 100.0 ± 0.00A| 576.93**     | —                    | —                    | —             |

Mean of mortality ± standard deviation within a column followed by the same letter did not differ significantly according to Tukey test (\( P = 0.05 \)); \( **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.
Table 6: Synergistic factor and cotoxicity index of the combination of the essential oils of E. camaldulensis and C. rigidus.

| Combinations | LC50 (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 mag against fourth-instar larvae of Aedes aegypti and found that, tested individually, C. inerme (LC50 = 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% (LC50 = 195.02 ppm) as well as in 50% V. negundo with 50% P. glabra (LC50 = 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 phyto compound 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 Rios 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 Dacyodes 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-allylansole, 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.
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