Research Article

Larvicidal Activities and Synergistic Effects of Essential Oils against Anopheles funestus and Culex quinquefasciatus (Diptera: Culicidae) from Kisumu, Kenya

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1. Introduction

Anopheles funestus remains one of the main malaria vectors in Sub-Saharan Africa but is poorly studied [1, 2]. This vector is an all-year-round vector in Kenya, Tanzania, and Ouganda [3] and seasonal vector in Burkina Faso and Sénégal [4, 5]. Bionomic traits and susceptibility to Plasmodium infection vary among the 13 An. funestus sibling species found throughout the Afrotropical region [3].

Culex quinquefasciatus is spread throughout the African tropical region and is the most abundant mosquito species in urban areas [6]. This species is a vector of bancroftian filariasis, Japanese encephalitis, St Louis encephalitis virus, West Nile virus, and Zika [7, 8]. Both vectors cause several million deaths and illnesses around the globe each year [9]. Mosquito-borne diseases in addition to having negative impact on the human health negatively affect the socio-economic status of the affected people. The current main approaches to reducing human-vector contact rely on the use of synthetic insecticides in the form of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [10]. These interventions have been successful in reducing disease burden and mosquito vector population in some African regions for the past years [11–13]. Unfortunately,
continuous use of synthetic insecticides in public health and agriculture has resulted in the development of resistance in mosquitoes [14, 15]. Moreover, such chemicals have caused serious environmental damage due to improper waste management [16, 17]. Therefore, new alternatives in developing additional control methods against target vector species that are sustainable and ecofriendly are urgently needed [18, 19].

Botanical insecticides are selective and biodegradable and have minor to no adverse effects on nontarget organisms and the environment [20]. They can be applied as larvicidal formulations and contributed to the elimination of malaria and continue to be evaluated for vector control in Africa [21]. In fact, Kenya is currently considering larvicidal applications to their list of vector control tools in the malaria-endemic regions. Temephos, fenitrothion, and diethylamphetamine that are recommended by the WHO [22] as larvicides are toxic and can cause health problems to humans as well as contaminate the environment [23]. The current chemical insecticides used are now threatened by the rapid rise in resistance due to the long-term use of these insecticides. Thus, new alternative mosquito control methods such as organic insecticides are urgently needed to replace synthetic insecticides.

Larvicidal activity of different botanical ingredients, e.g., essential oils (EOs), against different mosquito species is known [24–27]. Biosynthesized silver nanoparticles formed from Curcuma zedoaria EOs show a strong larvicidal activity against Cx. quinquefasciatus [28]. Combinations of different EOs are even more effective than single EOs. Benelli et al. [29] showed that combined EOs of Satureja montana and Aloysia citriodora caused mortality at a lethal concentration (LC50) of 18.3 μL L−1 against Cx. quinquefasciatus that was several times higher than single EOs. The efficacy of EOs, their formulation in nanoparticles, and their combination against Cx. quinquefasciatus were reported by several authors [30–33]. In addition, synergistic combinations of two or more EOs can overcome side effects associated with high doses of single EOs by decreasing the risk of resistance development, using smaller amounts of each compound, and affecting several targets simultaneously making resistance harder to develop than to each individual target [34–36].

In contrast with Cx. quinquefasciatus, not much is known on the susceptibility of An. funestus to EOs. Ntonga et al. [37] determined the toxicity of extracted oils of Ocimum canum, Ocimum basilicum, and Cymbopogon citratus against the larvae of An. funestus in Cameroon. These authors showed that O. canum and O. basilicum have insecticidal properties against adults and larvae of An. funestus [37–39]. Citrus fruit peels, pulp, and seeds were shown to have insecticidal activity against An. funestus [40].

This report determined the individual and combined toxicities of four EOs (from Lippia multiflora Moldenke, Lippia chevalieri Moldenke, Cymbopogon schoenanthus (L.) Spreng, and Lantana camara (L.) against the late 3rd–4th instar larvae of An. funestus and Cx. quinquefasciatus collected in western Kenya.

2. Material and Methods

2.1. Plant Sample Collection and Essential Oil Extraction. Plant leaves were collected in the vicinity of the "Institut de Recherche en Sciences Appliquées et Technologies" (IRSAT). EOs were extracted using hydrodistillation at the IRSAT in Ouagadougou, Burkina Faso. Four EOs from Lippia multiflora Moldenke, Lippia chevalieri Moldenke, Cymbopogon schoenanthus (L.) Spreng, and Lantana camara L. were distilled and were dried over anhydrous sodium sulphate and kept at 4°C.

2.2. Gas Chromatography Coupled with Mass Spectrometry Analysis of Essential Oils. The major and minor constituents of L. multiflora, C. schoenanthus, L. chevalieri, and L. camara EOs were identified and quantified using gas chromatography coupled with mass spectrometry analyses. Aliquots (20 μL) were removed from each essential oil sample using a micropipette, diluted at 1/5000 in hexane, and placed into a vial with an insert (VWR, Radnor, PA), allowing it to be injected into a GC-MS (Trace 1310; Thermo Fisher Scientific) equipped with a 30 m column (I.D. 0.25 mm, #36096–1420; Thermo Fisher Scientific). Helium was used as the carrier gas at a constant flow of 1 cc/min. Prepared samples were loaded into the GC-MS using an autosampler (TriPlus RSH; Thermo Fisher Scientific). The oven temperature was set at 45°C, held for 4 minutes followed by a heating gradient ramping to 230°C, and the 230°C temperature held for 6 minutes (total run: 28.5 min.). Chromatogram peaks were integrated using a Chromelone software MS quantitative processing method (Thermo Fisher Scientific), and the peaks were identified using the online NIST library. Major peaks found with consistently high abundances across multiple samples for each ornamental species were then recorded for comparison across plant ornamental species.

2.3. Dilution of Essential Oils. The EOs were diluted in ethanol, and the final concentrations were obtained from a stock solution of 10,000 ppm (0.5 mL of oil diluted in 49.5 mL of ethanol or 0.25 mL in 24.75 mL of ethanol) according to Table 1. To prepare mixtures, a stock solution (10,000 ppm) for two oil combinations were prepared by mixing 0.25 mL of each essential oil with 49.5 mL of absolute ethanol according to Muturi et al. [24].

2.4. Collection and Rearing of Mosquitoes. Female An. funestus resting indoor were captured with Prokopack in houses after verbal consent from household heads in the villages of Ratouro and Kadenge located in Siaya County in western Kenya. Cx. quinquefasciatus larvae were collected from rice fields in Ahero located in Kisumu County in western Kenya. Adult mosquitoes and larvae were placed in a cooler box, and adults were maintained on 10% sucrose
solution and transported to the insectary at the Centre for Global Health Research (KEMRI-CGHR) campus in Kisumu. Gravid female mosquitoes were transferred into cages (30 × 30 × 30 cm) with oviposition cups. Laid eggs were hatched in rainwater in small trays, and larvae were reared on a mixture of TetraMin (fish food) and brewer’s yeast that was provided daily in the insectary at a temperature of 26 ± 2°C and 70% to 80% relative humidity.

2.5. Larvicidal Assay. Larval bioassay tests to determine the larvicidal activity of single and binary EOs followed the WHO standard guidelines [41]. Twenty-five active early 3rd-4th instar larvae of An. funestus and Cx. quinquefasciatus were introduced into plastic cups containing rainwater in parallel. After 30 min in the cups, an appropriate quantity of various concentrations was added to the final volume of 200 mL, which final concentrations were 12.5, 25, 50, 75, and 100 ppm. Four replicates were tested at each concentration (N = 4). The control contained rainwater and absolute ethanol. Dead and moribund larvae were counted in each cup 24 h after exposure. According to the WHO protocol [41], larvae were recorded as dead if they could not move after probing the siphon or cervical region with a needle, while moribund larvae were recorded when they could not rise to the surface or were unable to show the characteristic diving reaction when the water was disturbed. The mortality rate was determined as the number of dead plus moribund larvae divided by the total number of larvae multiplied by 100.

2.6. Statistical Analyses. Lethal concentrations at 50% and 90% mortalities (LC50 and LC90) were determined using the XLStat 2016 software using a logistic regression using probit analysis, whereas means of larval mortality rate were calculated and compared using a Student–Newman–Keuls test with SAS 2009 software at P = 0.05. Abbott’s formula was used to correct for control mortality when mortality in the control groups was between 5% and 10% before probit analysis and ANOVA. Two-way ANOVA and Tukey’s test using R version 4.0.3 (2020-10-10) software was used to assess the effect of oil type and dose on the mortality of An. funestus and Cx. quinquefasciatus larvae. To examine the effect of oil mixtures, differences were compared to test the oil combinations for additivity. Mean mortality values for combination treatments were compared with those of single treatments. The effects were classified as additive if the difference was not significant, synergistic if the effect of EO combinations was significantly greater than the sum of their separate effects, and antagonistic if the effect of oil combinations was significantly lower than the sum of their separate effects.

3. Results

3.1. Chemical Composition and Yield of Essential Oils. Twenty-three compounds with concentrations higher than 0.1% were identified in L. multiflora EOs (Table 2). The principal constituents of this oil were caryophyllene (27.7%), germacrene D (9.8%), p-cymene (8.2%), humulene (6.7%), thymol (6.4%), and eucalyptol (5.3%). The other compounds were lower than 5% in this oil. In C. schoenanthus EOs, 25 compounds were identified (Table 2), and the main compounds were elemol (22.8%), α-eudesmol (19.9%), (+)-4-carene (14%), β-elemene (8.6%), and D-limonene (6.4). In L. camara EOs, 27 compounds were identified (Table 2), and the main compounds were caryophyllene (35%), carvophyllene oxide (14.8%), (+/-)-germacrene D (7.3%), and bicyclogermacrene (6.6%). In L. chevalieri EOs, fewer number of compounds were identified (Table 2), and the main compounds were caryophyllene (36.9%), germacrene D (25.6%), eucalyptol (9.1%), and humulene (5.5%).

3.2. Effect of Single Essential Oils. All the single EOs exhibited concentration-dependent larvicidal activity against the larvae of An. funestus and Cx. quinquefasciatus. Mortalities varied from 0 to 100% depending on the concentrations used on the larvae of both species and when single application of oils was used (Figures 1 and 2). At 100 ppm, EOs of C. schoenanthus and L. camara caused 100% mortality to An. funestus larvae. Among the single oils, L. camara and L. multiflora were more toxic against An. funestus with LC50s of 49.21 and 67.58 ppm, respectively, whereas C. schoenanthus was less toxic with an LC50 of 120.50 ppm (Table 3).

Single EOs of C. schoenanthus and L. multiflora showed mortalities of 100% at 50 ppm when tested with Cx. quinquefasciatus. Single testing of the EOs of L. multiflora and C. schoenanthus exhibited LC50s of 27.24 and 23.32 ppm, respectively, and the EOs were more toxic against the larvae of Cx. quinquefasciatus (Table 4). EOs of L. chevalieri were less toxic against Cx. quinquefasciatus exhibiting an LC50 of 54.11 ppm. Between the two species, Cx. quinquefasciatus was more susceptible than An. funestus to EOs according to mortalities.

3.3. Effect of Combined Essential Oils. The EO mixture from CS + LM was more toxic against An. funestus than other mixtures. The mortality produced by CS + LM EO mixture shows a curve that is above that of single oils with confidence intervals, which do not overlap (Figure 1). It was the only combination that showed synergistic activity between EOs. These two EOs have shown their synergistic activity
Table 2: Chemical composition and essential oil yields of plants.

| Compounds                  | R. time (min.) | L. multiflora | C. schoenanthus | L. camara | L. chevalieri |
|----------------------------|----------------|---------------|-----------------|-----------|--------------|
| α-Pinene                   | 12.3           | —             | —               | 2.6       | 0.8          |
| Camphene                   | 12.6           | —             | —               | 1.1       | 0.5          |
| β-Mycene                   | 13.2           | 2.4           | 0.3             | 2.1       | 1            |
| (+)-4-Carene               | 13.5           | —             | 14              | —         | —            |
| Myrtenyl acetate           | 13.6           | —             | —               | 0.6       | —            |
| β-Carene                   | 13.7           | —             | 0.3             | 2.7       | —            |
| p-Cymene                   | 13.9           | 8.2           | 0.7             | 0.6       | 1.1          |
| D-limonene                 | 14.1           | 5.3           | 1.2             | 4.7       | 9.1          |
| Eucalyptol                 | 14.1           | —             | 0.2             | 1.1       | 0.8          |
| trans-β-Ocimene            | 14.2           | 6.4           | —               | 1         | 0.4          |
| Terpineol                  | 15.1           | —             | 1.3             | —         | —            |
| Fenchone                   | 15.2           | —             | 0.3             | —         | —            |
| Linalool                   | 15.3           | 0.6           | —               | 1.1       | 0.4          |
| trans-p,2,8-Menthadien-1-ol| 15.9           | —             | 3.9             | —         | —            |
| (+)-2-Bornanone            | 16.2           | 1.4           | —               | —         | —            |
| Terpinen-4-ol              | 16.7           | 0.5           | 0.4             | 0.9       | 0.4          |
| α-Terpineol                | 17.1           | —             | 1.0             | —         | —            |
| trans-Piperitol            | 17.2           | —             | 1.3             | —         | —            |
| Thymol                     | 18.4           | 5.4           | —               | 0.9       | —            |
| Carvacrol                  | 18.5           | —             | 0.4             | 0.5       | —            |
| γ-Elemene                  | 19.3           | —             | —               | 1.1       | 2.2          |
| Copaene                    | 19.4           | 1.4           | —               | 1.9       | —            |
| β-Elemene                  | 19.9           | —             | 8.6             | —         | —            |
| γ-Gurjunene                | 20.1           | —             | 0.8             | —         | —            |
| Caryophyllene              | 20.3           | 27.7          | 6               | 35        | 36.9         |
| ω-Gurjunene                | 20.6           | —             | 1.3             | —         | —            |
| γ-Muurole                  | 20.7           | —             | 0.4             | —         | —            |
| cis-β-Farnesene            | 20.8           | 3.1           | —               | —         | —            |
| (E)-β-farnesene            | 20.9           | —             | —               | —         | —            |
| β-Longipinene              | 20.9           | —             | 0.3             | —         | —            |
| Humulene                   | 21.1           | 6.7           | 0.9             | —         | 5.5          |
| Germacrene D               | 21.3           | 9.8           | —               | —         | 25.6         |
| β-Selinene                 | 21.4           | —             | 2.9             | —         | —            |
| (+/-)-Germacrene D         | 21.5           | —             | —               | 7.3       | —            |
| γ-Muurole                  | 21.6           | 0.3           | —               | —         | —            |
| Bicyclergemacrene          | 21.7           | —             | —               | 6.6       | —            |
| β-Guaiene                  | 21.8           | 0.5           | 1.8             | —         | —            |
| α-Panasinsen               | 21.9           | —             | —               | —         | —            |
| β-Acorenol                 | 22             | —             | —               | —         | —            |
| Elemol                     | 22.1           | —             | 22.8            | —         | —            |
| Caryophyllene oxide        | 22.3           | 5.2           | 1.6             | 14.8      | 2.2          |
| (−)-Spathulenol             | 22.8           | —             | —               | 1.9       | —            |
| Aromandendrene             | 22.9           | —             | —               | 0.3       | 0.3          |
| Cubenol                    | 23.3           | 0.2           | —               | —         | —            |
| α-Eudesmol                 | 23.7           | —             | 19.9            | —         | —            |
| Geranyl-α-terpinene         | 24.5           | 0.2           | —               | —         | —            |
| Isoaoromandendrene epoxide  | 24.9           | —             | —               | 1.0       | —            |
| α-Vetivol                  | 26.1           | 0.3           | —               | —         | —            |
| m-Camphorene               | 26.8           | 0.3           | —               | —         | —            |
| p-Camphorene               | 27.2           | 0.2           | —               | —         | —            |
| 1-Heptatriacotanol         | 27.3           | 0.3           | —               | 0.3       | 0.3          |
| Total yield identified     | 91.6           | 97.5          | 97.5            | 96.7      | —            |

R. time = retention time.
Figure 1: Continued.
Figure 1: Larval mortality rate with confidence limits of the 3rd–4th instar larvae of *Anopheles funestus* following exposure to various concentrations of essential oils and their mixtures. LM: *Lippia multiflora*; LCh: *Lippia chevalieri*; LC: *Lantana camara*; CS: *Cymbopogon schoenanthus*.

Figure 2: Continued.
with respect to An. funestus (Table 5). Mortality with combined EOs was 100% at 100 ppm, while the single EOs of CS exhibited 75% mortality and LM 30% mortality (Figure 1). The other combinations exhibited additive activity because there is no difference with at least one of the single EOs. The LCa + LM ($p = 0.0001$) and LCa + LCh ($p = 0.00001$) combinations were antagonists with low toxicity of their mixture against An. funestus compared with single EO application.

The EO mixtures of LCh + CS ($p = 0.00001$), LM + LCh ($p = 0.00001$), and LCa + LCh ($p = 0.00001$) exhibited synergistic effect against Cx. quinquefasciatus (Table 6).
Table 3: The 50% and 90% lethal concentrations (LC50 and LC90, respectively), their 95% confidence intervals, and regression parameters of the larvicidal activity of essential oils against Anopheles funestus 24 h post treatment.

| Plants     | LC50 (ppm) | LLC-ULC | LC90 (ppm) | LLC-ULC | Chi²(Wald) | p-value |
|------------|------------|---------|------------|---------|------------|---------|
| LM         | 67.58      | (61.55–78.86) | 109.07     | (100.43–120.30) | 140.52     | p < 0.0001|
| LCh        | 105.74     | (91.38–122.97) | 175.235    | (152.21–213.83) | 45.75      | p < 0.0001|
| LCa        | 49.21      | (39.38–57.82)  | 91.26      | (82.22–102.19)  | 108.01     | p < 0.0001|
| CS         | 120.50     | (108.22–144.19) | 172.00     | (147.23–226.10) | 25.99      | p < 0.0001|

LC: lethal concentration; LCL: lower confidence limit; UCL: upper confidence limit; ppm: parts per million; LM: Lippia multiflora; LCh: Lippia chevalieri; LCa: Lantana camara; CS: Cymbopogon schoenanthus.

Table 4: The 50% and 90% lethal concentrations (LC50 and LC90, respectively), their 95% confidence intervals, and regression parameters of the larvicidal activity of essential oils against Culex quinquefasciatus 24 h post treatment.

| Plants     | LC50 (ppm) | LLC-ULC | LC90 (ppm) | LLC-ULC | Chi²(Wald) | p-value |
|------------|------------|---------|------------|---------|------------|---------|
| LM         | 27.24      | (10.45–33.44) | 35.76      | (26.16–39.15) | 18.72      | p < 0.0001|
| LCh        | 54.11      | (20.80–25.72) | 49.21      | (39.38–57.82) | 111.34     | p < 0.0001|
| LCa        | 38.54      | (34.15–42.45) | 62.14      | (56.51–71.08) | 51.65      | p < 0.0001|
| CS         | 23.32      | (20.80–25.72) | 34.25      | (31.34–38.40) | 65.02      | p < 0.0001|

LC: lethal concentration; LCL: lower confidence limit; UCL: upper confidence limit; ppm: parts per million; LM: Lippia multiflora; LCh: Lippia chevalieri; LCa: Lantana camara; CS: Cymbopogon schoenanthus.

Table 5: ANOVA analysis comparing the response of Anopheles funestus larvae to treatment with EO combinations and single EOs.

| Treatment | Estimate | Std. Error | t value | p-value | Effect |
|-----------|----------|------------|---------|---------|--------|
| CS + LCa  | −0.1109  | 0.0526     | −2.1098 | 0.0393  | Additive|
| CS + LM   | 0.2959   | 0.0458     | 6.4582  | 0.0001  | Synergist|
| LCa + LCh | −0.1812  | 0.0438     | −4.134  | 1.00E-04| Antagonist|
| Lca + LCh | −0.0976  | 0.0321     | −3.0438 | 0.0035  | Antagonist|
| LCh + CS  | −0.0483  | 0.0245     | −1.9707 | 0.0536  | Additive|
| LM + LCh  | −0.0415  | 0.0337     | −1.232  | 0.223   | Additive|

LM: Lippia multiflora; LCh: Lippia chevalieri; LCa: Lantana camara; CS: Cymbopogon schoenanthus.

Table 6: ANOVA contrasts comparing the response of Culex quinquefasciatus larvae to EO combinations relative to individual oils.

| Treatment | Estimate | Std. Error | t value | p-value | Effect |
|-----------|----------|------------|---------|---------|--------|
| CS + LCa  | 0.0424   | 0.0529     | 0.8005  | 0.4268  | Additive|
| CS + LM   | 0.0794   | 0.0569     | 1.397   | 0.1678  | Additive|
| LCh + LCh | 0.1342   | 0.0282     | 4.7588  | 0.0001  | Synergist|
| LCh + LM  | 0.1279   | 0.0483     | 2.6494  | 0.0104  | Additive|
| CS + LCh  | 0.0305   | 0.0581     | 5.256   | 0.00001 | Synergist|
| LM + LCh  | 0.2268   | 0.0466     | 4.8674  | 0.00001 | Synergist|

LM: Lippia multiflora; LCh: Lippia chevalieri; LCa: Lantana camara; CS: Cymbopogon schoenanthus.

These three mixtures exhibited more than 90% mortality of Cx. quinquefasciatus larvae compared with the others (Figure 2). The other combinations (CS + LCa, CS + LM, and LCa + LCh) showed additive effects against Cx. quinquefasciatus larvae with a low average of mortality (Table 6).

LC50 and LC90 confirmed the synergistic effect of CS + LM on An. funestus. These concentrations were 44.05 ppm (34.87–51.66) and 86.92 ppm (79.59–95.48), respectively (Table 7). The LC50 of the oils applied individually were 67.58 ppm (61.55–78.86) and 120.50 ppm (108.22–144.19), respectively, for LM and CS (Table 3). L. camara was individually more toxic to An. funestus, but its combination with the other oils showed an additive effect against larvae of An. funestus except with L. chevalieri oil where an antagonistic effect was recorded. Indeed, the LC50s were 127.90 ppm (106.77–159.01) (Table 7), 49.21 ppm (39.38–57.82), and 105.74 ppm (91.38–122.97) for LCa + LCh, LCa, and LCh (Table 3), respectively.

Cx. quinquefasciatus is more susceptible to EOs, exhibiting a low LC50 compared with An. funestus, and no antagonism of the EOs was observed. On the other hand, additive and synergistic effects were observed respectively with CS + LCa, CS + LM, and LCa + LM and LCa + LCh, LCh + CS, and LM + LCh (Table 6). Single EOs exhibited LC50 values of 27.24 ppm (10.45–33.44), 54.11 ppm (43.36–63.74), 38.54 ppm (34.15–42.45), and 23.32 ppm (20.80–25.72), respectively, for LM, LCh, LCa, and CS (Table 4). The LC50 and LC90 of the combinations were lower than those of the single EOs, thus showing the additive effect or the synergistic effect (Tables 4 and 8). The LCa + LCh
dependent and varied with different mosquito species. While the larvicidal activity of essential oils mixture against *An. funestus* was most active against 91.2 ppm, and 144.5 ppm, respectively. Our results show that EOs from *L. multiflora* are most toxic against *An. funestus* followed by EOs from *L. chevalieri*, and *C. schoenanthus*. The variable effects of EOs on *An. funestus* larvae were also reported by Ntonga et al. [37] who showed that *C. citratus* EO is most active against *An. funestus* larvae, followed by EOs from *O. canum* and *O. basilicum*, with LC50 values for stage IV larva of 34.6 ppm, 91.2 ppm, and 144.5 ppm, respectively.

Similarly, *C. quinquefasciatus* larvae are more affected by EOs from *L. multiflora*, followed by EOs of *C. citratus* because of different larval behaviour in the aquatic environment. *An. funestus* larvae are very active in the water, exhibiting fearful movements by avoiding the presence of surface film [47]. They sink quickly in water when disturbed and stay under water for a long period [2, 48]. This behaviour helps them to avoid contact with insecticidal compounds that form surface films like plant EOs. On the other hand, *C. quinquefasciatus* larvae cannot stay underwater for long periods, and thus they frequently break the water surface and contact insecticidal compounds that form a surface film. On the other hand, *An. funestus* larvae stay longer on the bottom and thus avoid surface films. Since the EOs are volatile, the majority of the applied EOs evaporate rapidly, diminishing the surface toxicity and allowing *An. funestus*, which remained submerged for a long time to escape the brunt of the surface toxicity and stay alive, compared with *C. quinquefasciatus* that break the surface more frequently. In addition, the differences in the thickness of the cuticle of the two mosquito larvae also explain why *C. quinquefasciatus* is more susceptible to the EOs. Indeed, the *An. funestus* at our study area in west Kenya are highly resistant to surface insecticides [49], exhibiting a thicker cuticle [50] than that of *C. quinquefasciatus*.

4. Discussion

The EOs used in this study contain a mixture of major and minor compounds. Caryophyllene, caryophyllene oxide, and eucalyptol are the major compounds found in all the EOs. These compounds have already been identified in the EOs of *L. camara*, *C. schoenanthus*, and *L. multiflora* in Burkina Faso [42] and have been used in the present study. Bicyclogermacrene and (+/-)-germacrene D have been previously demonstrated in *L. camara* oils [43]. For *L. chevalieri* EOs, germacrene D and humulene were the predominant compounds. Germacrene D was already demonstrated in these oils in 2007 in Burkina Faso [44]. In *C. schoenanthus* oils, (+)-4-carene, β-elemene, elemol, and α-eudesmol are the major compounds. Elemol and α-eudesmol have been identified in oils from the same plant for antibacterial control [45].

In *L. multiflora* EOs, p-cymene, γ-terpinene, and thymol were identified as the major components [42]. Some differences, however, exist between the composition of our EOs and other published results of EOs of the same plants. These differences can be due to the geographical location of the collected plants, the period of the year when these plants were harvested, the extraction methods, and the parts of the plants used for extracting the EOs [46].

All the EOs that we tested exhibited larvicidal activities on *An. funestus* and *C. quinquefasciatus*. Mortality was dose dependent and varied with different mosquito species. *Culex quinquefasciatus* was more susceptible to EOs than *An. funestus* because of different larval behaviour in the aquatic environment. *An. funestus* larvae are very active in the water, exhibiting fearful movements by avoiding the presence of surface film [47]. They sink quickly in water when disturbed and stay under water for a long period [2, 48]. This behaviour helps them to avoid contact with insecticidal compounds that form surface films like plant EOs. On the other hand, *C. quinquefasciatus* larvae cannot stay underwater for long periods, and thus they frequently break the water surface and contact insecticidal compounds that form a surface film. On the other hand, *An. funestus* larvae stay longer on the bottom and thus avoid surface films. Since the EOs are volatile, the majority of the applied EOs evaporate rapidly, diminishing the surface toxicity and allowing *An. funestus*, which remained submerged for a long time to escape the brunt of the surface toxicity and stay alive, compared with *C. quinquefasciatus* that break the surface more frequently. In addition, the differences in the thickness of the cuticle of the two mosquito larvae also explain why *C. quinquefasciatus* is more susceptible to the EOs. Indeed, the *An. funestus* at our study area in west Kenya are highly resistant to surface insecticides [49], exhibiting a thicker cuticle [50] than that of *C. quinquefasciatus*.

Our results show that EOs from *L. camara* are most toxic against *An. funestus* followed by EOs from *L. multiflora*, *L. chevalieri*, and *C. schoenanthus*. The variable effects of EOs on *An. funestus* larvae were also reported by Ntonga et al. [37] who showed that *C. citratus* EO is most active against *An. funestus* larvae, followed by EOs from *O. canum* and *O. basilicum*, with LC50 values for stage IV larva of 34.6 ppm, 91.2 ppm, and 144.5 ppm, respectively.

Similarly, *C. quinquefasciatus* larvae are more affected by EOs from *L. multiflora*, followed by EOs of
C. schoenanthus and EOs of L. camara and L. chevalieri. Benelli et al. [51] compared 8 EOs that they tested against Cx. quinquefasciatus larvae and found that EOs of Cinnamomum verum was most active (LC50 = 40.7 µL L−1), followed by Lippia alba (LC50 = 59.6 µL L−1), Ocimum basilicum (LC50 = 68.6 µL L−1), Mentha spicata (LC50 = 88.2 µL L−1), and Achillea ligustica (LC50 = 89.5 µL L−1). Therefore, the EOs that affect larvae are dose dependent, species dependent, and chemical-specific compounds that differ from one EO to another [43, 51, 52]. Compounds such as thymol and 1,8-cineol [53], eugenol [54], carvacrol, β-citronellol, geraniol, and linalool show different specificities and effects [36, 55]. In addition to exhibiting larvicidal activity for each individual EO extracted from each plant, combined EOs show enhanced toxicities and several show synergistic effects against mosquito larvae [24, 31, 56, 57].

Our study shows that extracted plant EOs exhibited synergistic, antagonistic, and additive effects. Using EO combinations showed that An. funestus was less susceptible than Cx. quinquefasciatus. Only the combination of the EOs from CS + LM exhibited a synergistic effect against the larvae of An. funestus. The other EO combinations were either antagonistic or additive.

Cx. quinquefasciatus was more susceptible to EO combinations from LCa + LCh, LCh + CS, and LM + LCa, suggesting that the EOs are synergistically more effective against Cx. quinquefasciatus, whereas the rest of the EO combinations were additive. Similar effects have been reported for larvae and adults of Cx. quinquefasciatus [24, 29, 31, 46]. EOs from Allium sativum (bulbs) combined with those from Citrus paradisi (leaves) have strong larvicidal properties against Cx. quinquefasciatus [31]. These same EOs are even more effective against Cx. quinquefasciatus when combined with temephos [31]. Our results show that the effects of EOs from LCa + LCh are species specific and they affect Cx. quinquefasciatus but not An. funestus. This phenomenon can be partially explained by the behaviour of the An. funestus larvae in the water [48] because the plant EOs are volatile, staying underneath the water for long time increases larval survival by avoiding the contact with the EOs film and also diminishing the amount of the toxic film because of rapid evaporation of the organic layer.

Very little information is available on the effect of EOs on An. funestus. Most studies with EOs use malarial vectors such as Anopheles gambiae, Anopheles stephensi, and Anopheles cracens, and fewer studies reported the effect of combination of EOs on these mosquitoes including the synergistic effect of combined EOs [58–60].

Our study, therefore, provides information for the first time on the lethal effect of EOs on An. funestus, one of the major vectors of malaria in Africa.

We would like to hypothesize that the synergistic and antagonistic effects of EOs are probably due to the formation of additional new molecules after these EOs were combined, which enhanced their effects on the tested larvae. The enhanced activity may also be due to the simultaneous effects on different targets, enhancing the larvicidal effect of the tested EOs up to tenfold [36, 61], severely impacting larval survival [62]. Synergistic and antagonistic effects have also been demonstrated on Ae. aegypti and Culex pipiens [24, 36]. The EOs used in this study exhibiting larvicidal effects contain caryophyllene, thymol, germacrene D, eucalyptol, elemol, and α-eudesmol. These compounds of the EOs are known larvicides against mosquitoes when applied as single extract or in combination of extracts [29, 36, 63]. Cheng et al. [64] found that leaf EOs from Cryptomeria japonica (Thunb. ex L. f.) D. Don (LC50 = 28.4 mg/L) were more toxic to Ae. aegypti larvae than its major constituents, 16-kaurene (LC50 = 57.0 mg/L) and elemol (LC50 > 100.0 mg/L), both of which are present in the samples at 20%. The authors suggested that the minor compounds 3-carene (LC50 = 25.3 mg/L), terpinene (LC50 = 32.1 mg/L), α-terpinene (LC50 = 28.1 mg/L), and y-terpinene (LC50 = 26.8 mg/L) that are also present in our EOs contributed to the larvicidal activity [64].

Similarly, the combination of carvacrol and thymol exhibits synergistic effects against Cx. pipiens larvae [36]. Sarma et al. [35] showed that the best larvicidal composition was obtained when limonene was mixed with diallyl disulfide against Ae. aegypti larvae. The combination of EOs with permethrin and deltamethrin increased the effectiveness of these mixtures compared with the product taken individually [65]. These compounds inhibit detoxification enzymes such as cytochrome P450 and glutathione S-transferase of Ae. aegypti and An. gambiae [65]. Both the major and minor compounds found in the EO mixtures affect larval and adult mosquitoes’ nervous system, their digestive tract, and the larval cuticle [46].

5. Conclusion

This is the first report using combined EOs from L. camara, L. multiflora, L. chevalieri, and C. schoenanthus against An. funestus and Cx. quinquefasciatus larvae. Our results show that Cx. quinquefasciatus is highly susceptible compared with An. funestus. In both species, single EOs were toxic and combined EOs showed synergistic toxic and antagonist effects. Extracted EOs from CS + LM are effective against An. funestus, whereas extracted EOs from LCa + LCh, LCh + CS, and LM + LCh were effective against Cx. quinquefasciatus. We also showed that larval behaviour is important when EOs are evaluated.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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