Hindawi
Psyche: A Journal of Entomology
Volume 2022, Article ID 8302696, 13 pages
https://doi.org/10.1155/2022/8302696

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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Received 20 September 2021; Revised 10 February 2022; Accepted 15 February 2022; Published 8 March 2022

Academic Editor: Luciano Toma

Rapid development of resistance in vector mosquitoes to synthetic insecticides is a major challenge for malaria control. The use of plant-derived essential oils (EOs) is an attractive strategy in controlling mosquito populations because they are environmentally safe and may have a lower chance of developing resistance. This study assessed the larvicidal activities of EOs from *Lantana camara*, *Lippia multiflora*, *Lippia chevalieri*, and *Cymbopogon schoenanthus* against *Anopheles funestus* and *Culex quinquefasciatus*. The 3rd–4th instar larvae were tested using a World Health Organization (WHO)-modified protocol to evaluate larval mortality 24 h after exposure to EOs and their binary combinations. *Culex quinquefasciatus* larvae were more susceptible to EOs than *An. funestus* larvae. For *Cx. quinquefasciatus,* the lethal concentrations at 50% mortality (LC_{50}s) of EOs from *C. schoenanthus*, *L. multiflora*, *L. camara*, and *L. chevalieri* were 23.32, 27.24, 38.54, and 54.11 ppm, respectively; whereas for *An. funestus*, the EO LC_{50}s were 120.5, 67.5, 49.21, and 105.74 ppm, respectively. Synergistic effects were observed using EOs from *C. schoenanthus* + *L. multiflora* (LC_{50} = 44.05 ppm) on *An. funestus*, while *L. camara* + *L. chevalieri* (LC_{50} = 33.16 ppm), *L. chevalieri* + *C. schoenanthus* (LC_{50} = 12.08 ppm), and *L. multiflora* + *L. chevalieri* (LC_{50} = 20.61 ppm) were synergistic for *Cx. quinquefasciatus*. These results indicate the potential of EOs derived from local plants and their binary combinations as botanical larvicides. The EOs could be used as future ecofriendly agents to control these vectors.

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, fenthion, and diflubenzuron 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 (LC_{50}) 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 3^{rd}–4^{th} 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 Chromeleon 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

Table 1: Essential oils' dilution procedure.

Final concentration (ppm)	Final volume (mL)	Stock solution (ppm)	Initial volume (mL)	Rainwater volume (mL)
12.5	200	10,000	0.25	199.75
25	200	10,000	0.5	19.5
50	200	10,000	1	199
100	200	10,000	2	198

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 3^{rd}–4^{th} 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 (LC_{50} and LC_{90}) 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%), caryophyllene 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 LC_{50}s of 49.21 and 67.58 ppm, respectively, whereas *C. schoenanthus* was less toxic with an LC_{50} 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 LC_{50}s 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 LC_{50} 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.)	Percentage of each compound			
		L. multiflora	*C. schoenanthus*	*L. camara*	*L. chevalieri*
α-Pinene	12.3	—	—	2.6	0.8
Camphene	12.6	—	—	1.1	0.5
β-Myrcene	13.2	2.4	0.3	2.1	1
(+)-4-Carene	13.5	—	14	—	—
Myrtenyl acetate	13.6	—	—	0.6	—
3-Carene	13.7	—	0.3	2.7	—
p-Cymene	13.9	8.2	0.7	0.6	1.1
D-limonene	14	1.5	6.4	2.5	1.9
Eucalyptol	14.1	5.3	1.2	4.7	9.1
trans-β-Ocimene	14.2	—	0.2	0.8	—
α-Phellandrene	14.4	3.9	0.4	0.3	4.6
β-Ocimene	14.5	—	—	1.1	3.7
γ-Terpinene	14.7	6.4	—	1	0.4
Terpineol	15.1	—	—	1.3	—
Fenchone	15.2	—	0.3	—	—
Linalool	15.3	0.6	—	—	0.3
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	—
Copaene	19.4	1.4	—	1.9	2.2
β-Elemene	19.9	—	8.6	—	—
γ-Gurjunene	20.1	—	—	0.8	—
Caryophyllene	20.3	27.7	6	35	36.9
β-Gurjunene	20.6	—	1.3	—	—
γ-Muurolene	20.7	—	0.4	—	—
cis-β-Farnesene	20.8	3.1	—	—	—
(E)-β-famesene	20.9	—	—	—	—
β-Longipinene	20.9	—	—	0.3	—
Humulene	21	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-Muurolene	21.6	0.3	—	—	—
Bicyclogermacrene	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	—	—	—
Isoaromadendrene 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
Total yield identified		91.6	97.5	97.5	96.7

R. time = retention time.

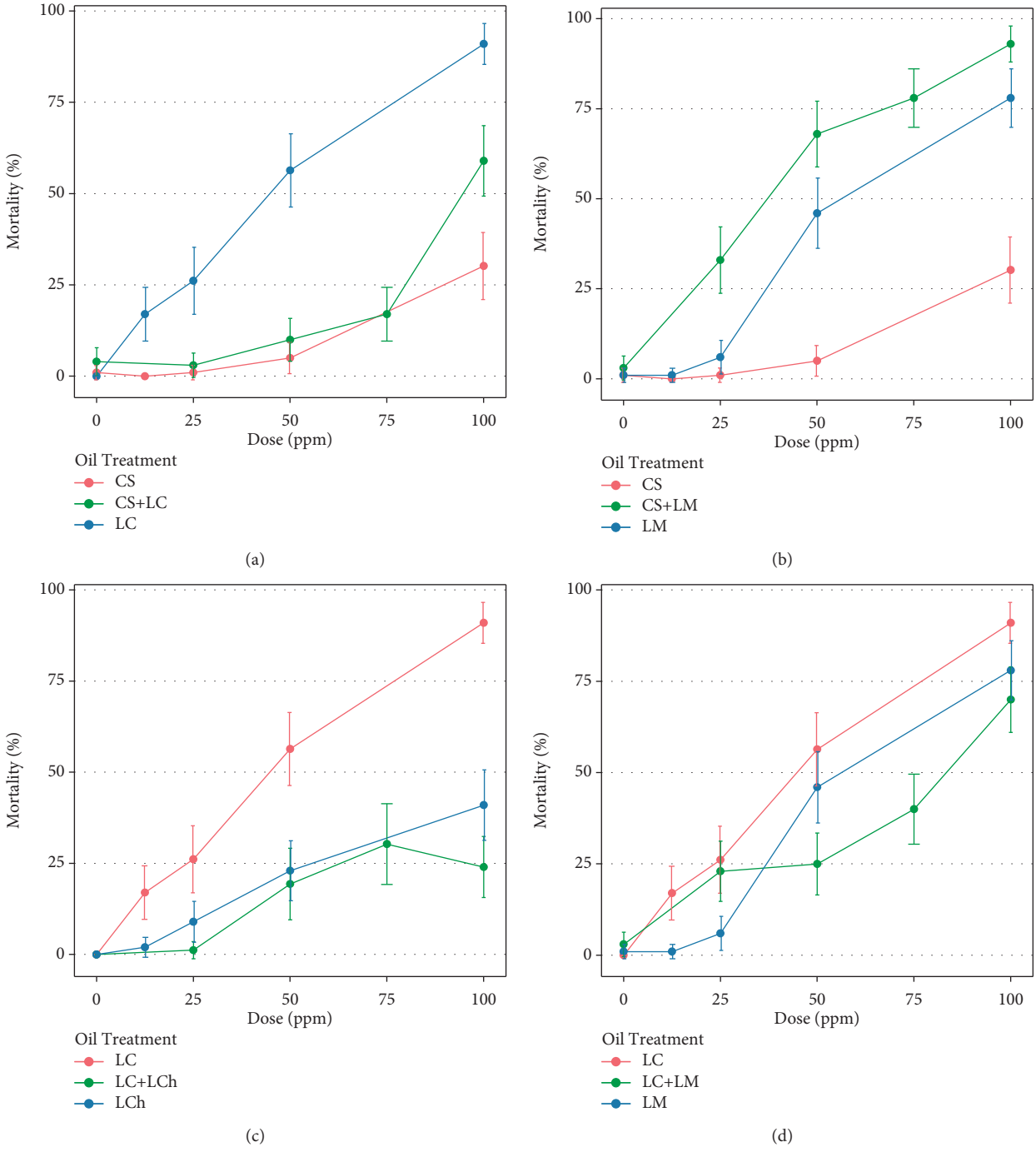

Figure 1: Continued.

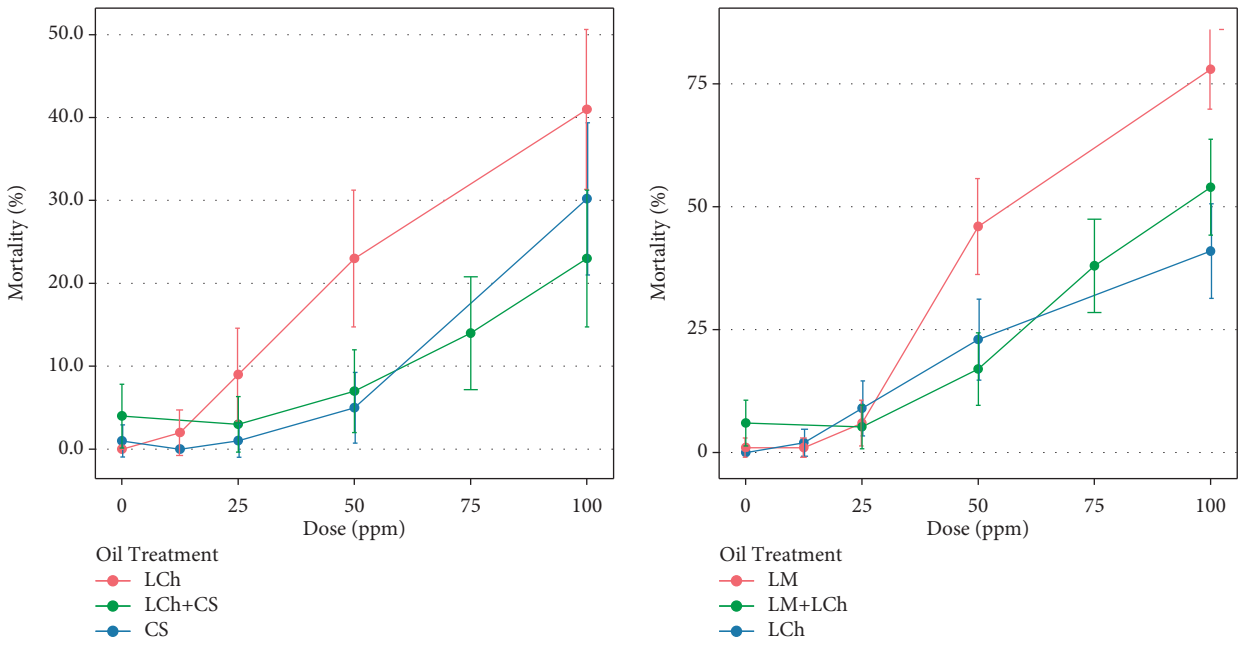

Figure 1: Larval mortality rate with confidence limits of the 3rd–4th instar larvae of *Anopheles funestus* following exposure to various concentrations of essential oils and their mixtures. *LM*: *Lippia multiflora*; *LCh*: *Lippia chevalieri*; *LCa*: *Lantana camara*; *CS*: *Cymbopogon schoenanthus*.

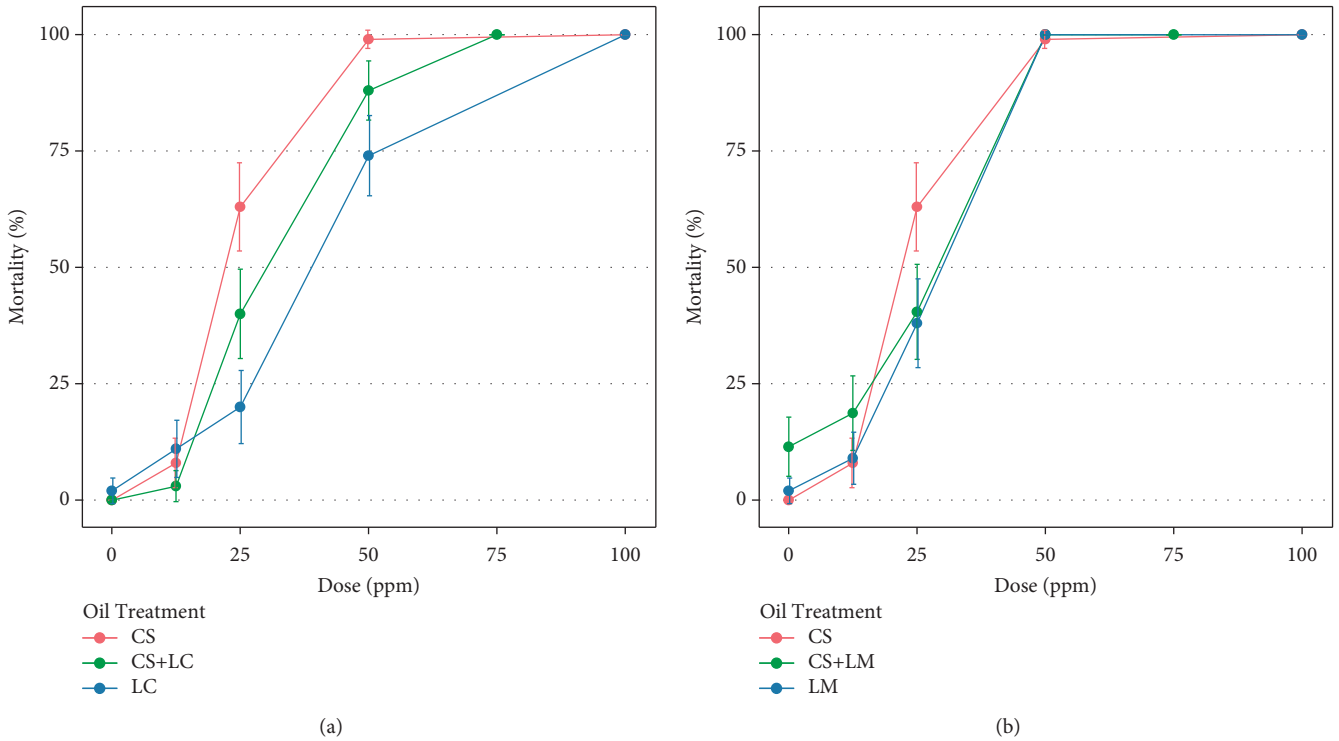

Figure 2: Continued.

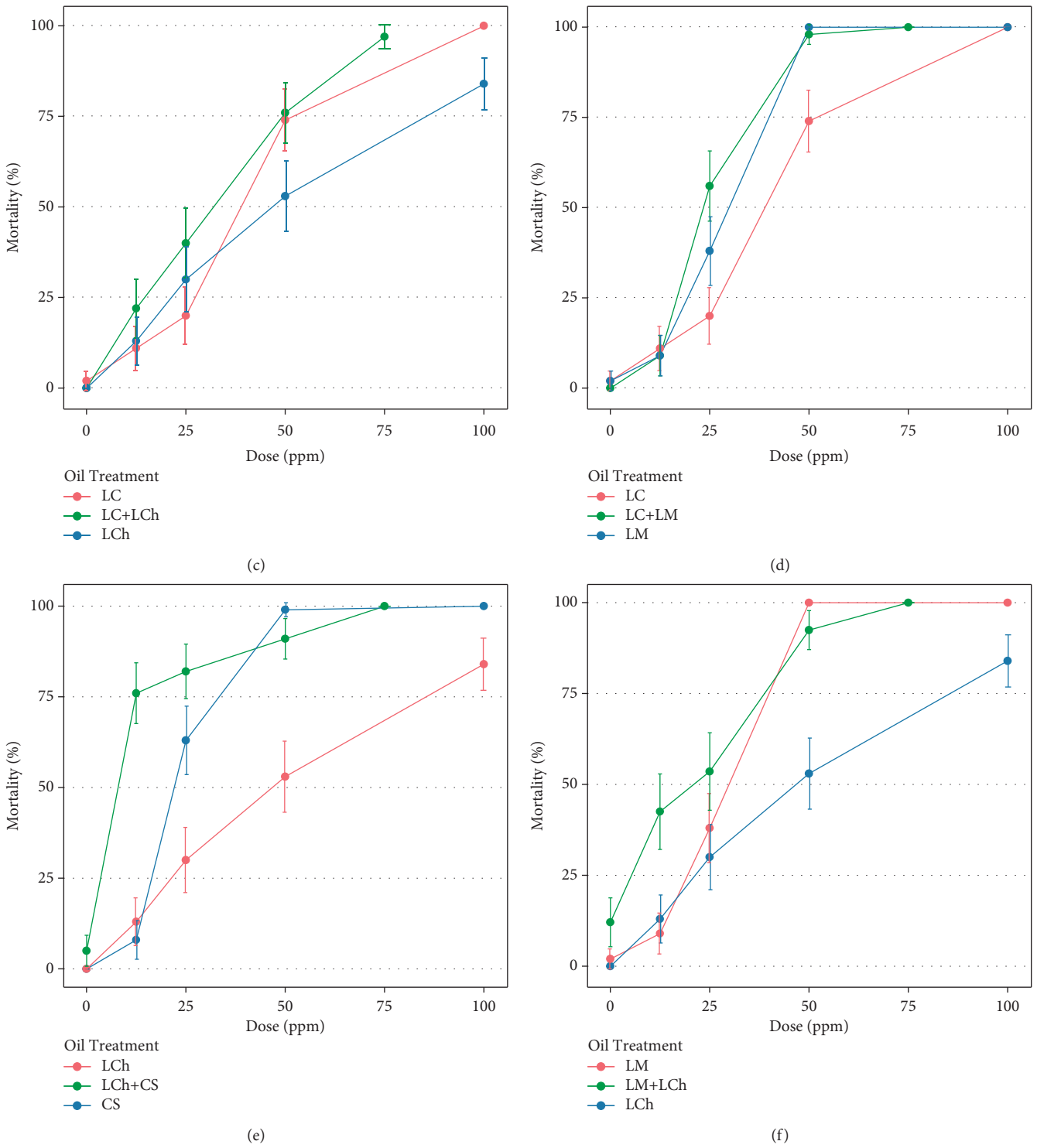

Figure 2: Larval mortality rate on the 3rd–4th instar larvae of *Culex quinquefasciatus* after exposure to various concentrations of essential oils and their mixtures. *LM*: *Lippia multiflora*; *LCh*: *Lippia chevalieri*; *LCa*: *Lantana camara*; *CS*: *Cymbopogon schoenanthus*.

($p = 0.0001$) 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 $LCa + LM$ ($p = 0.0035$) 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 (LC_{50} and LC_{90}, respectively), their 95% confidence intervals, and regression parameters of the larvicidal activity of essential oils against *Anopheles funestus* 24 h post treatment.

Plants	LC_{50} (ppm)	LLC-ULC	LC_{90} (ppm)	LLC-ULC	Chi^2(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 (LC_{50} and LC_{90}, respectively), their 95% confidence intervals, and regression parameters of the larvicidal activity of essential oils against *Culex quinquefasciatus* 24 h post treatment.

Plants	LC_{50} (ppm)	LLC-ULC	LC_{90} (ppm)	LLC-ULC	Chi^2(Wald)	*p*-value
LM	27.24	(10.45–33.44)	35.76	(26.16–39.15)	18.72	$p < 0.0001$
LCh	54.11	(43.36–63.74)	102.45	(92.01–115.24)	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	1,00*E*-04	Antagonist
LCa + *LM*	−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
LCa + *LCh*	0.1342	0.0282	4.7588	0.0001	Synergist
LCa + *LM*	0.1279	0.0483	2.6494	0.0104	Additive
LCh + *CS*	0.3053	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* + *LM*) showed additive effects against *Cx. quinquefasciatus* larvae with a low average of mortality (Table 6).

LC_{50} and LC_{90} 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 LC_{50}s 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 LC_{50}s 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 LC_{50} 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 LC_{50} 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 LC_{50} and LC_{90} 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*

Table 7: The 50% and 90% lethal concentrations (LC_{50} and LC_{90}, respectively), their 95% confidence intervals, and regression parameters of the larvicidal activity of essential oil mixture against *Anopheles funestus* 24 h post treatment.

Plants	LC_{50} (ppm)	LLC-ULC	LC_{90} (ppm)	LLC-ULC	Chi^2(Wald)	p-value
LM + LCa	79.22	(30.24–90.62)	144.65	(128.25–179.46)	23.17	$p < 0.0001$
LM + CS	44.05	(34.87–51.66)	86.92	(79.59–95.48)	121.04	$p < 0.0001$
LM + LCh.	92.93	(85.20–102.66)	146.22	(129.13–180.46)	30.88	$p < 0.0001$
LCh + CS	140.75	(121.22–193.51)	216.11	(173.47–352.48	13.41	$p < 0.0001$
LCh + LCa	127.90	(106.77–159.01)	206.02	(171.51–271.99)	31.76	$p < 0.0001$
CS + LCa	100.92	(78.28–147.32)	156.59	(119.63–231.38)	0.00	$p < 0.0001$

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 8: The 50% and 90% lethal concentrations (LC_{50} and LC_{90}, respectively), their 95% confidence intervals, and regression parameters of the larvicidal activity of essential oils mixture against *Culex quinquefasciatus* 24 h post treatment.

Plants	LC_{50} (ppm)	LLC-ULC	LC_{90} (ppm)	LLC-ULC	Chi^2 (Wald)	p-value
LM + LCa	24.96	(22.01–27.80)	37.93	(34.59–42.34)	88.76	$p < 0.0001$
LM + CS	24.12	(21.27–26.78)	43.42	(39.07–50.26)	0.00	$p < 0.0001$
LM + LCh	20.61	(08.61–28.03)	46.16	(40.33–52.53)	42.23	$p < 0.0001$
LCh + CS	12.08	(5.68–17.76)	36.50	30.62–43.47	108.11	$p < 0.0001$
LCh + LCa	33.16	(26.51–38.83)	60.31	(54.79–66.64)	120.57	$p < 0.0001$
CS + LCa	31.99	(28.52–35.32)	48.93	(45.15–53.54)	137.38	$p < 0.0001$

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*.

combination gave opposite results depending on the species. This combination was antagonistic to *An. funestus* (Table 5), while to *Cx. quinquefasciatus* it was synergistic (Table 6).

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 *Cx. 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, *Cx. 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 *Cx. 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 *Cx. 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 *Cx. 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 was 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 LC_{50} values for stage IV larva of 34.6 ppm, 91.2 ppm, and 144.5 ppm, respectively.

Similarly, *Cx. 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 ($LC_{50} = 40.7\,\mu L\,L^{-1}$), followed by *Lippia alba* ($LC_{50} = 59.6\,\mu L\,L^{-1}$), *Ocimum basilicum* ($LC_{50} = 68.6\,\mu L\,L^{-1}$), *Mentha spicata* ($LC_{50} = 88.2\,\mu L\,L^{-1}$), and *Achillea ligustica* ($LC_{50} = 89.5\,\mu L\,L^{-1}$). 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 ($LC_{50} = 28.4$ mg/L) were more toxic to *Ae. aegypti* larvae than its major constituents, 16-kaurene ($LC_{50} = 57.0$ mg/L) and elemol ($LC_{50} > 100.0$ mg/L), both of which are present in the samples at 20%. The authors suggested that the minor compounds 3-carene ($LC_{50} = 25.3$ mg/L), terpinolene ($LC_{50} = 32.1$ mg/L), α-terpinene ($LC_{50} = 28.1$ mg/L), and γ-terpinene ($LC_{50} = 26.8$ 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.

Acknowledgments

This work was supported by The World Academy of Science grant (IsDB-TWAS)008/2019 to Dimitri W. Wangrawa. The authors are grateful to the Entomology Department of the Kenya Medical Research Institute (KEMRI), Kisumu, and its technicians for accepting the laboratory work and for

helping during bioassays. The authors thank Dr. Chloé Lahondere of Virginia Tech for the identification of the essential oil compounds.

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