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RESEARCH ARTICLE

Chemical composition and toxicity of commercial *Mentha spicata* and *Eucalyptus citriodora* essential oils on *Culex quinquefasciatus* and non-target insects

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Abstract

Current vector control strategies based on synthetic chemicals are not eco-friendly against non-target organisms; hence, alternative approaches are highly required. Commercially purchased oil of *Mentha spicata* (Spearmint) and *Eucalyptus citriodora* (Citriodora) were examined against the medical pest *Cx. quinquefasciatus* (Say) and their non-toxicity on the aquatic species was evaluated. Chemical screening with gas chromatography coupled with mass spectrometry (GC–MS) analysis revealed a total of 14 and 11 compounds in Citriodora and Spearmint oils, respectively, with the highest peak (%) at carvone (70.44%) and isopulegol (30.4%). The larvicidal activity on the fourth instar larvae of *Cx. quinquefasciatus* showed dose-dependent mortality and significance at a 100 ppm concentration 48 h post-treatment with Citriodora (76.4%, $P \leq 0.001$) and Spearmint (100%, $P \leq 0.001$). Additionally, the photomicrograph of the fourth instar larvae revealed significant physical abnormalities in the head and midgut tissues post-exposure to Spearmint and Citriodora oils. Moreover, the histological assay revealed severe damage in the epithelial cells and gut lumen 2 to 24 h post-treatment. The repellency percentage of adult *Culex* mosquitoes was prominent across both oils at 150 ppm 210 min post-exposure. Non-target toxicity on the aquatic predator showed both essential oils (Spearmint oil (17.2%) and Citriodora oil (15.2%)) are safer at the maximum treatment (200 ppm) compared to temephos (75.4% at 1 ppm). The in silico screening of phyto-compounds derived by both essential oils with BeeTox (online server) showed no contact toxicity to the honey bee *Apis mellifera*. Overall, the present research revealed that Spearmint and Citriodora essential oils and their active phyto-compounds were toxic to *Cx. quinquefasciatus* and harmless to the aquatic predator and honey bee.

Keywords Essential oils · *Mentha spicata* · *Eucalyptus citriodora* · Vector control · Histopathology · Non-target

Introduction

Mosquitoes, vectors of diseases like dengue and malaria, pose global health and economic challenges (Giunti et al. 2023). Malaria alone has caused over 247 million cases, with a mortality rate exceeding 90%, particularly affecting young children in sub-Saharan Africa (Tolle 2009; Benelli et al. 2020a; Paton et al. 2021). Moreover, climate change is expected to expand the *Aedes* mosquito species, elevating the risk of dengue for 4.7 billion people by 2070 (Malavige et al. 2023). Despite efforts, implementing effective mosquito control strategies, especially in the field, remains challenging (Benelli and Senthil-Nathan 2019; Benelli et al. 2020b). Diseases causing mild to severe disorders that are transmitted by *Culex* mosquitoes highlight the need for targeted control measures (Mazzara et al. 2023). Targeting aquatic and immobile larval mosquitoes offers a promising approach (Fillinger and Lindsay 2011). However, the widespread use of synthetic larvicides faces challenges like environmental harm and resistance (Agathokleous et al. 2023a; Benelli 2019; Haddi et al. 2023). As resistance grows, the effectiveness of commercial pesticides against filarial vectors diminishes, potentially increasing disease transmission

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(Hafez 2023). These identified challenges prompted us to seek an environmentally and health-centered alternative for mosquito vector control (Agathokleous et al. 2023b). Natural plant-based insecticides have emerged as a promising solution in the quest for effective alternatives (Benelli et al. 2020b). Plant-derived secondary molecules, serving as chemical defenses against herbivorous insects, exhibit properties such as feeding deterrence, insect growth influence, and toxic effect induction on mosquitoes (Dubey et al. 2010; Benelli et al. 2019). Many botanicals with insecticidal effects contain volatile oils, known as essential oils (EOs), found in plant parts like leaves, flowers, stems, and roots (Pavela 2015). These EOs, characterized by their volatility and aromatic nature, contribute to the distinctive scent and flavor of various plants (Pavela 2013, 2014).

Commercial EOs, versatile for various applications like sprays, lotions, candles, and diffusers, offer individuals the flexibility to choose the method that aligns with their preferences and needs (Osanloo et al. 2017; Benelli et al. 2018a, b). Possessing natural properties such as antibacterial, leishmanicidal, and larvicidal effects, as well as repellent activity, EOs are not only selectively effective against target organisms but also environmentally degradable and non-toxic to non-target species, making them favorable for mosquito larvae control (Osanloo et al. 2018). Existing research presents diverse studies on mosquito vector management using EOs (Khanavi et al. 2010; Vasantha-Srinivasan et al. 2017, 2018; Chellappandian et al. 2018a, b). Due to the qualitative and quantitative variations in chemical content, EOs exhibit changing toxicity against different dengue vectors (Cheng et al. 2003). The larvicidal actions of EOs are attributed to complex mixtures of volatile compounds, including terpenes, phenols, aldehydes, and esters (Benelli et al. 2018a, b). Current research emphasizes bio-rational plants from diverse regions and their insecticidal properties (Radhakrishnan et al. 2023a, b). While past literature explored the larvicidal potential of EOs, mechanistic insights into their actions on arthropod vectors remain unclear (Benelli and Senthil-Nathan 2019; Aziz et al. 2023; Kalvikkarasan et al. 2023; Vasantha-Srinivasan et al. 2024).

Mentha spicata (Spearmint), a member of the Lamiaceae family and native to Europe and Asia, is now cultivated globally, favoring temperate climates and thriving in moist habitats (Hudz et al. 2023). Known for its aromatic leaves containing EOs, including carvone, Spearmint is utilized for culinary and medicinal purposes, particularly in treating respiratory and digestive issues (de Araujo Moysés et al. 2023). *Eucalyptus citriodora*, part of the *Eucalyptus* genus in the Myrtaceae family and native to Australia, has been introduced and cultivated in various climates worldwide, including Asia, Africa, and the Americas (Amri et al. 2023). The EO derived from its leaves is particularly rich in citronellal and contributes to its lemon scent, making it valuable in the fragrance industry and as an ingredient in insect repellents (Khedhri et al. 2023). Both Spearmint and Citriodora EOs are recognized as environmentally friendly alternatives to synthetic insecticides. The importance of testing non-target toxicity against beneficial organisms such as *Toxorhynchites splendens* (commonly known as the mosquito fish or gambusia) is crucial in the development and use of pesticides or other control methods (Pavela et al. 2019; Vasantha-Srinivasan et al. 2023).

Overall, the objectives of the present investigation were as follows: (i) the chemical characterization of commercial Spearmint (*M. spicata*) and Citriodora (*E. citriodora*) oils using the gas chromatography-mass spectrometry (GC–MS) technique; (ii) exploring the toxic larvicidal action of EOs on the medical pest *Cx. quinquefasciatus*; (iii) detecting the midgut toxicity of EOs on the fourth instar larvae of the filarial vector; (iv) estimating the repellent actions of EOs on the adult *Culex* mosquito; and (v) In-vitro and in-silico screening of the impact of EOs on the aquatic mosquito predator and honey bee.

Materials and methods

Based on the previous research on EOs with insecticidal properties, the two vital essential bio-active oils, Citriodora (*E. citriodora*) and Spearmint (*M. spicata*), were designated. These two EOs were obtained commercially from Katyani Exports India Pvt. Ltd. (Delhi, India) and studied for their larvicidal activity and impact on the histological aspects of the midgut of the larval intestine of *Cx. quinquefasciatus*. The commercially purchased oils were cosmetic grade (100%), and the volatile oil was acquired through steam distillation of the herbal leaves and then further packed into sterile glass containers.

Rearing of mosquitoes

The *Cx. quinquefasciatus* mosquito culture for the present study was maintained in the lab without exposure to pesticides and vectors. In addition, the cultures were maintained at specific laboratory conditions (27 ± 2 °C, relative humidity [RH], and 75–85% with a 14 h:10 h light/dark (L:D) photoperiod). Brewer's yeast and dog biscuits in a proportion of 1:5 were provided as food to the larvae that emerged from the eggs. The pupae that emerged from the larvae were collected and placed in a plastic container with 250 mL of water. This plastic container was then kept in a 60 cm × 60 cm × 60 cm breeding cage covered with a nylon net for adult emergence. Next, Petri dishes with cotton swabs containing a 10% sucrose solution and wet raisins (dried grapes) were placed in the breeding cages for the emerging mosquitoes to eat. After 3 days of mosquito emergence, the female mosquitoes were fed with sucrose for 6 h and then delivered to a hen that was kept inside the mosquito

breeding cage for blood feeding throughout the night. The fourth instar larvae were then used for conducting experiments. Our earlier research protocol was used for the insect-rearing methods (Chellappandian et al. 2018b).

Identification of essential oil components by GC–MS

The isolated EOs were dissolved using ethyl alcohol at a 1:1 ratio. Next, 2 µL of the samples were dissolved in high-performance liquid chromatography (HPLC)-grade methanol and subjected to JEOL GC mate II GC–MS (Agilent Technologies 6890N Network GC system (Mumbai, India)) equipped with a secondary electron multiplier. The column (HP5) was then fused with silica (50 m × 0.25 mm ID). The chemical characterization of the EO was adapted from the previous methodology (Vasantha-Srinivasan et al. 2018). The identification of the compounds was assessed through GC attached to a mass spectrometer. Afterward, the EO chemical structure was investigated by interpreting the GC–MS mass spectrum through the National Institute of Standard and Technology (NIST) database.

Preparation of test solutions for larvicidal activity

One gram of each test EO was transferred into a 100 mL standard volumetric flask and made up with ethanol. From this stock solution, serial volumes such as 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, and 10.0 mL of the solution were pipetted into other flasks and chlorine-free tap water was added to obtain 25, 50, 75, and 100 ppm of solution. This solution was then used for the larvicidal bioassay.

Larvicidal bioassay

The larvicidal bioassay of the EOs was conducted following the World Health Organization (2005) process with minor alterations. The fourth instar larvae were kept inside disposable plastic cups (200 mL) containing 100 mL of dechlorinated water using 25, 50, 75, and 100 ppm of EOs. During the treatment period, the larval diet was supplied in individual treatment cups, specifically if a significant mortality rate in the control was recorded. The conditions remained uninterrupted for 24 h, and the rate of mortality was logged 48 h post-treatment. The dead larvae numbers were identified at the initial experiment stages (0 and 24 h). The experiments were replicated five times, and each replication set included a control treated with an aqueous solution of dimethyl sulfoxide (DMSO (0.5%)). Larvicidal activity that displayed at least 50% mortality (lethal concentration $(LC)_{50}$) and 90% mortality (LC_{90}) within 48 h was estimated and considered for further experiments. The statistical analyses of the larvicidal assays were conducted based on the methodology of Finney (1971) Probit. In addition, the mortality (%) in the treatments was calculated with the formula adapted from Abbott's (1925) formula (1 and 2).

$$\text{Percentage of mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100 \quad (1)$$

$$\text{Corrected percentage of mortality} = \left(1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}}\right) \times 100 \quad (2)$$

Histological analysis

For the histological tests, the treated and control newly ecdysed fourth instar larvae of *Cx. quinquefasciatus* were separated from the laboratory culture raised with the larval diet and later incorporated with Citriodora (100 ppm) and Spearmint (75 ppm) oil. Twenty-four hours later, larval survival was observed. Afterward, the larvae were fixed in the bouins reagent post-exposure (6, 12, 24, and 48 h) for 24 h. Further experiments were conducted following the adapted protocol of Senthil-Nathan et al. (2008).

Repellent assay

The repellent activities of both EOs on the adult *Culex* mosquitoes treated with diverse doses (25, 50, 75, and 100 ppm) were analyzed with the improved procedure (Chellappandian et al. 2019). The whole investigation was reviewed and permitted by the institution ethical committee board (Manonmaniam Sundaranar University, Tirunelveli, India). Previously mated (5–6 days) post-emerged female gravid mosquitoes (100 total) were starved for 1 day without any blood meal in the mosquito cages (45 cm × 35 cm × 5 cm). A discernible strike with a marker pen was made on a fixed area (4 cm × 12 cm) on the forearm of every three human volunteers while the rest of the arm was protected with sleeves of paper. As a control, 0.5% DMSO was placed on one forearm of the volunteer following a similar procedure. The *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes were tested from 18.00 h to 02.00 h and 06.00 h to 14.00 h, respectively. The mosquitoes that landed on the forearm were considered and those on the hands were shaken off before they sucked the blood. The percentage of repellency was estimated using the following adapted formula (3):

$$\% \text{ Repellency} = [(\text{Ta} - \text{Tb})/\text{Ta}] \times 100 \quad (3)$$

where *Ta* represents the mosquito numbers in the control group, and *Tb* represents the mosquito numbers in the treatment group.

BeeTox toxicity prediction

The BeeTox is an artificial intelligence (AI) open online tool utilized to predict the acute toxicity of ligands/chemicals on honey bees (Moreira-Filho et al. 2021). In the present study, the free BeeTox online servers were used to predict the bee toxicities of the phyto-compounds of both Spearmint (11 chemicals) and Citriodora (14 chemicals) EOs identified through the GC–MS technique. Furthermore, the AI tool was designed to divide the compounds into two independent datasets according to the honey bee exposure type (contact and oral). The entire experimental protocol was adapted based on that of Moreira-Filho et al. (2021).

Non-target toxicity assay

The effects of Spearmint and Citriodora EOs on the aquatic predator and beneficial species *Toxorhynchites splendens* (Theobald) were determined using the previously adapted protocol (Yogarajalakshmi et al. 2020). The authentication of the aquatic predator species was given by the zoologist of Manonmaniam Sundaranar University, Tirunelveli, India. The non-target species were retained in the same ecological habitat of the aquatic mosquito larvae (dengue and filarial vector), which were isolated in separate tanks with water (47 cm diameter and 27 cm depth) at 27 ± 2 °C and 75% RH, and fed with the second instar larvae of *Cx. quinquefasciatus*. The aquatic predator (*Tx. splendens*) was exposed to different dosages (50, 100, 150, and 200 ppm) of both EOs, and the obtained results were related to the toxicity of the synthetic chemical Temephos at the 1.0 ppm dosage. In the individual treatment, a total of 20 replications were executed, and five replicates were used as a control treatment (without any chemicals). The mortality (%) was experimental 24 h post-treatment.

Statistical analyses

The mortality data of the experiment were assessed using analysis of variance (ANOVA of arcsine, logarithmic, and square root transformed percentages), and the results were expressed as a mean value of five replicates. Similarly, Tukey's multiple range test (significance at $p < 0.05$) was used to analyze significant differences between the treatments using the Minitab®16 software program. The SigmaPlot version 11 of the MicroCal software was utilized for plotting the line and bar graphs. The lethal dosages necessary to develop 50 and 90% mortality (LC_{50} and LC_{90}) in the larvae 24 h post-treatment were examined utilizing Probit analysis with an interval dependability (95%) using the Minitab®16 statistical software.

Results

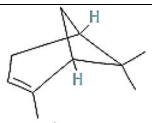
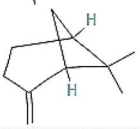
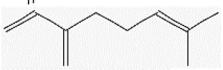
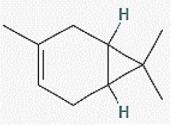
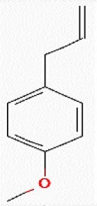
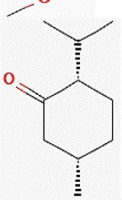
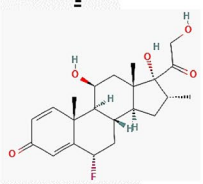
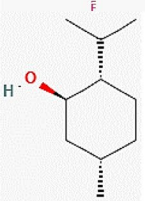
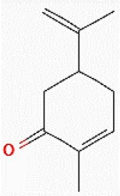
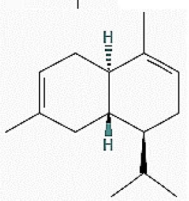
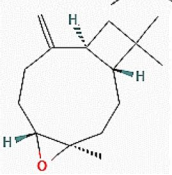
Identification of chemical components in the essential oils

The EO is screened through GC–MS to identify the major components responsible for the insecticidal activity of the *Cx. quinquefasciatus* larvae. In the Spearmint oil, the GC–MS analysis of its components revealed about 11 components (Table 1). Out of these, the major components were estragole (22.7%) and carvone (70.44%), and the minor components were β-pinene (3.41%) and α-pinene (1.43%). The chromatogram is presented in (Fig. 1A). The GC–MS analysis of Citriodora oil revealed 14 identified compounds (Table 2 and Fig. 1B). The chief ingredients of *Eucalyptus citriodora* oil were citronellal (9.34%), citronellol (17.39%), isopulegol (30.44%), and 2,6-Octadiene 2,6, dimethyl. The minor components identified were x-pinene (1.35%), eucalyptol (1.75%), caryophyllene (4.59%), 4-bromo-n-butyl (3.36%), and 2-methylhexacosane (2.88%).

Larvicidal activity

The larvicidal actions of Citriodora EO 24 h post-treatment displayed reliant mortality activity on the fourth instar larvae of *Cx. quinquefasciatus*. The mortality percentage was significant at the maximum dosage (100 ppm) with 76.4% ($F_{4,20} = 41.31$, $P \leq 0.001$) 24 h post-treatment, whereas 25 ppm caused 30.2% mortality (Fig. 2A). Similarly, at 48 h post-treatment with Citriodora (100 ppm), a prominent mortality rate of 83.5% ($F_{4,20} = 18.54$, $P \leq 0.001$) was seen, though it was not significant with the other treatment dosages of 75 ppm (73.1%, $F_{4,20} = 13.76$, $P \leq 0.001$) and 50 ppm (67.9%, $F_{4,20} = 19.99$, $P \leq 0.001$), respectively (Fig. 2B). Correspondingly, the larvicidal action of Spearmint EO displayed a significant mortality rate across 24 and 48 h of treatment against the fourth instar larvae. The maximum dosage (100 ppm) only produced an 18.77% ($F_{4,20} = 19.55$, $P \leq 0.001$) mortality rate 24 h post-treatment (Fig. 2C). Despite 100 ppm Spearmint oil causing 100% ($F_{4,20} = 17.88$, $P \leq 0.001$) mortality 48 h post-treatment, it was significant with the other treatment dosages of 50 ppm (45.21%, $F_{4,20} = 19.33$, $P \leq 0.001$) and

Table 1 Compounds identified in the Spearmint essential oil through GC-MS analysis

Sl.No.	Compound Identified	R.T	Peak area (%)	Structure
1	α-pinene	5.96	1.43	
2	β-Pinene	6.72	3.41	
3	β-myrcene	7.31	0.23	
4	3-carene	7.40	0.20	
5	Estragole	7.68	22.7	
6	Isomenthone	10.09	0.09	
7	Paramenthasone	10.19	0.08	
8	Isomenthol	10.32	0.14	
9	Carvone	11.26	70.44	
10	δ-cadinene	14.83	0.08	
11	Caryophyllene oxide	15.22	0.11	

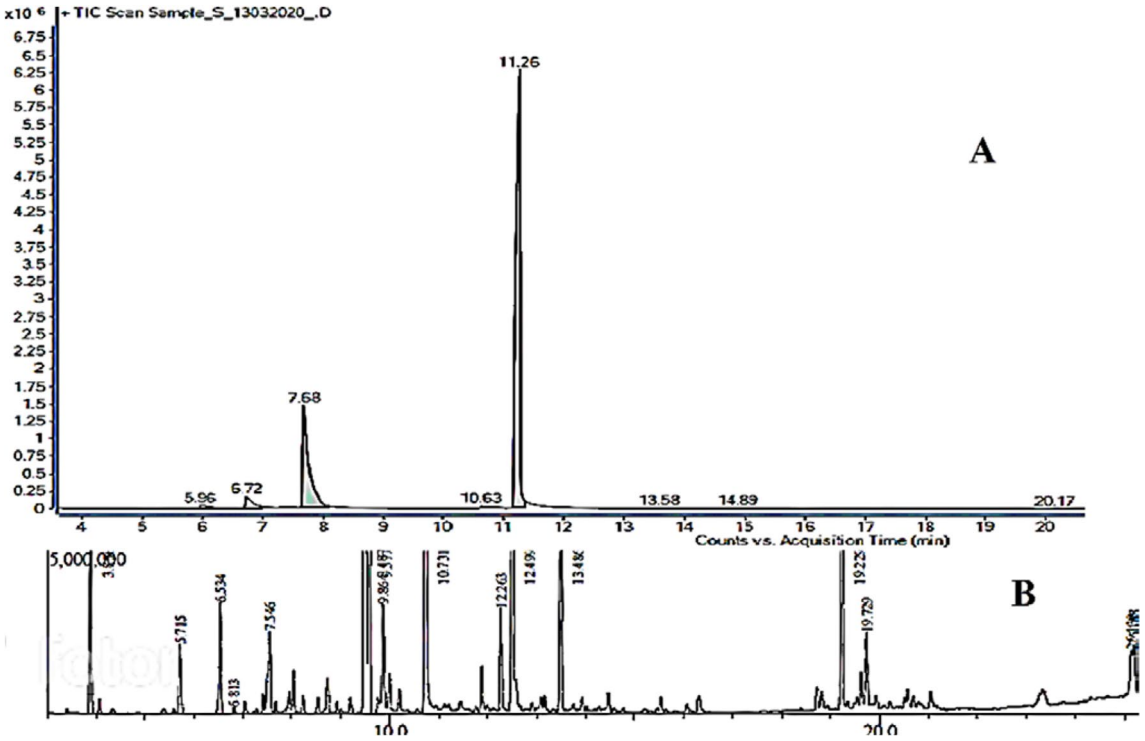


Fig. 1 GC–MS chromatogram (retention time and peak area) of **A** Spearmint oil and **B** Citriodora essential oil

25 ppm (39.11%, $F_{4,20} = 15.61$, $P \leq 0.001$), respectively (Fig. 2D). The LC_{50} values of Spearmint oil against the fourth instar larvae of the filarial vector were seen at 51.00 ppm (24 h) and 32.99 ppm (48 h), while the LC_{90} values were recorded at 146.5 ppm (24 h) and 133.64 ppm (48 h). Correspondingly, the Citriodora oil delivered LC_{50} values at 45.36 ppm (24 h) and 29.15 ppm (48 h), and LC_{90} values at 214.5 ppm (24 h) and 196.3 ppm (48 h) (Table 3). Notably, all EOs tested delivered an LC_{50} value > 500 ppm 48 h post-treatment and were considered as effective larvicides. The confidence intervals (95%) of the larvae mortality percentage were also determined. Overall, the results showed that the Citriodora and Spearmint oils were effective if their confidence interval did not overlap and were significantly different.

Photomicrography analysis

The fourth instar larvae of the filarial vector showed significant abnormalities in their midgut and head position post-exposure (24 h) to Citriodora and Spearmint oils (Fig. 3). Moreover, the midgut epithelial layer, gut lumen, and anal segments were severely damaged by the Citriodora oil treatment (Fig. 3B) as compared to that of the control (Fig. 3A). Similarly, the head, anal, and midgut segments collapsed following the Spearmint oil treatment (Fig. 3C), whereas the control appeared normal with a clear head, midgut tissues, and anal segment layers.

Histological studies

The mid-gut of the arthropod larvae was subdivided into two different regions, each including one characteristic cell type. Depending on their stage of development, the clear cells displayed different degrees of apical swelling into the gut lumen, reducing intercellular contacts with the neighboring cells and displaying nuclei and brush border degeneration, as shown in the *Cx. quinquefasciatus* control (Fig. 4H). Dark cells showed normal intercellular contacts along the whole lateral plasma membranes, normal nuclei, a well-developed brush border, and a normal adhesive basal lamina, as observed in the control sections of *Cx. quinquefasciatus*.

Four hours post-treatment

When compared with the control group (Fig. 4I), the Citriodora-treated larvae did not show much change. In the Spearmint-treated larvae, even after the 4-h treatment, there were signs of gut wall and epithelial cell degeneration and non-distinct larval segments when compared with the control. In addition, the peritrophic membrane also started to undergo degeneration (Fig. 4E and F).

Table 2 Compounds identified in the Citriodora essential oil through GC–MS analysis

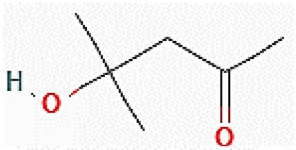
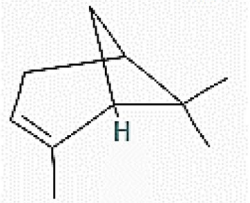
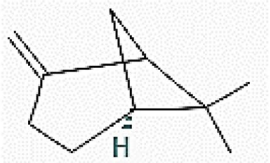
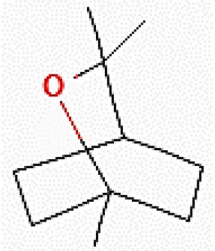
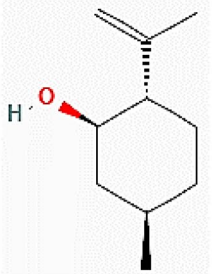
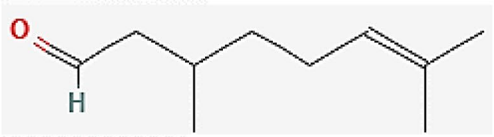
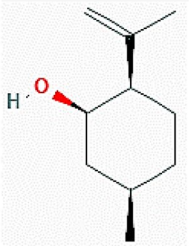
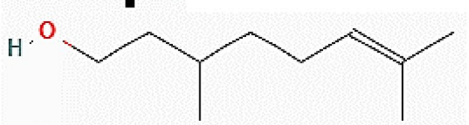
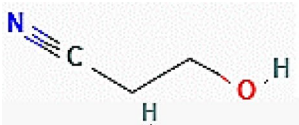
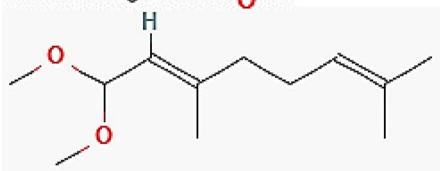
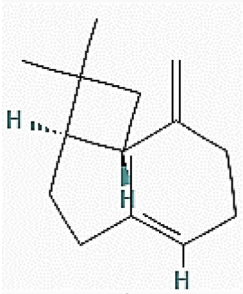
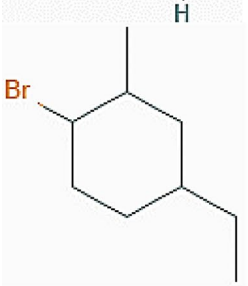
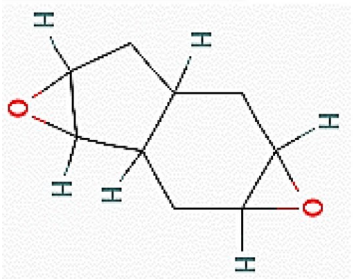
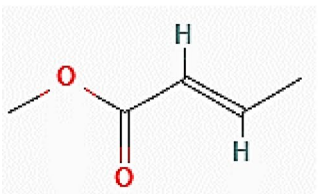
Sl. No.	Compound Identified	R.T	Peak Area%	Structure
1	2-Pentanone, 4-hydroxy-4-methyl-	3.88	4.49	
2	Alpha Pinene	5.71	1.35	
3	(5S)-6,6-dimethyl-2-methylidenebicyclo[3.1.1] heptane	6.53	2.25	
4	Eucalyptol	7.54	1.75	
5	Isopulegol	9.48	30.44	
6	Citronellal	9.57	9.34	
7	(1R,2R,5R)-5-methyl -2-(prop-1-en-2-yl) cyclohexanol	9.86	2.41	
8	Citronellol	10.73	17.39	

Table 2 (continued)

9	3-Hydroxypropanenitrile	12.26	2.06	
10	2,6 Octadiene 2,6 dimethyl	12.49	11.08	
11	Caryophyllene	13.48	4.59	
12	5-(1-bromo-1-methyl -ethyl)-2 methyl cyclo hexanone	19.22	7.24	
13	Bicyclononadiene diepoxide	25.13	2.46	
14	Crotonic acid methyl ester	25.73	1.81	

Eight hours post-treatment

The analysis of the anterior mid-gut showed a progression of swollen clear cells, vacuoles, and degenerated nuclei in both oil treatments, with the effect being more pronounced in the Citriodora-treated larvae (Fig. 4B). In the posterior mid-gut, disruption of the junctional complexes among the dark cells progressed apically, together with their cytoplasmic and nuclear lysis, local detachment from the basal lamina, and degeneration of microvilli. In the Spearmint-treated larvae, the epithelial cells were collapsed. Moreover, the epithelial cells showed signs of degeneration when compared to those of the control (Fig. 4B and I).

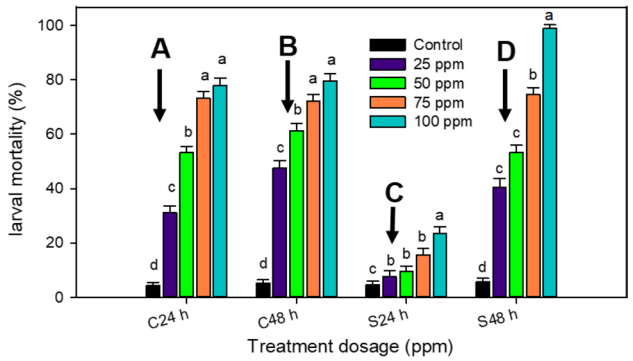


Fig. 2 Mortality of different essential oils against IV instar *Cx. quinquefasciatus*. **A** Citriodora essential oil treatment post 24 h. **B** Citriodora essential oil treatment post 48 h. **C** Spearmint essential oil treatment post 24 h. **D** Spearmint essential oil treatment post 48 h. Means (±SEM) followed by the same letters above bars indicate no significant difference ($P \leq 0.05$) according to a Tukey's test

Sixteen hours post-treatment

Treatment with either EO was followed by severe degeneration of the larval gut cells. Additionally, there was complete degeneration of the peritrophic membrane and epithelial cells in the Citriodora EO treatment (Fig. 4A–C). The epithelial cells showed signs of degeneration in the Citriodora EO-treated larvae though the peritrophic membrane was visible (Fig. 4C).

Twenty-four hours post-treatment

Maximum epithelial cells are vacuolated and degenerated. Gut-histological examination results revealed significant toxicity with EOs among the two diverse parts of the mid-gut epithelium based on the exposure period. The key cyto-pathological changes were outsized vacuoles of dissimilar sizes with damaged membranes at the epithelial cell's apical sides, wide cellular damage to the peritrophic membrane and gut lumen, and disruption of the brush border cells. Additionally, larval segmentation was completely lost, indicating heavy necrosis, and the nucleus showed complete dissolution.

Repellent activity

The repellency percentage was significant against both the Spearmint and Citriodora oil treatments, up to the maximum protection time of 210 min. The Spearmint oil treatment delivered a maximum protection percentage (98.4%, $F_{4,20} = 27.33$, $P \leq 0.001$) up to the maximum protection period of 210 min (Fig. 5A). In addition, the Citriodora EO treatment delivered a significant repellent percentage

Table 3 LC_{50} and LC_{90} value of different essential oil against *Cx. quinquefasciatus* for 24 h (ppm) and 48 h (ppm). Control, nil mortality. Significant at $P < 0.05$ level. LC_{50}, lethal concentration that kill 50% of the larval population; LC_{90}, lethal concentration that kill 90% of the larval population; UCL, upper confidence limit; LCL, lower confidence limit; ×2, chi-square

Essential oil	Exposure time (h)	LC_{50} (ppm)	95% Fiducial confidence limit		LC_{90} (min)	95% Fiducial confidence limit		Slope	Intercept	R^2	Chi-test (χ^2) sig
			Lower	Upper		Lower	Upper				
Spearmint	24	51.00	36.30	71.66	146.51	104.27	205.85	2.83	0.16	0.85	0.649
	48	32.99	17.83	61.11	47.36	133.64	458.18	1.48	2.76	0.63	0.336
Citriodora	24	45.36	27.66	78.75	214.5	131.39	350.34	1.89	1.86	0.99	0.993
	48	29.15	15.85	53.59	196.36	106.8	360.99	1.55	2.72	0.96	0.967

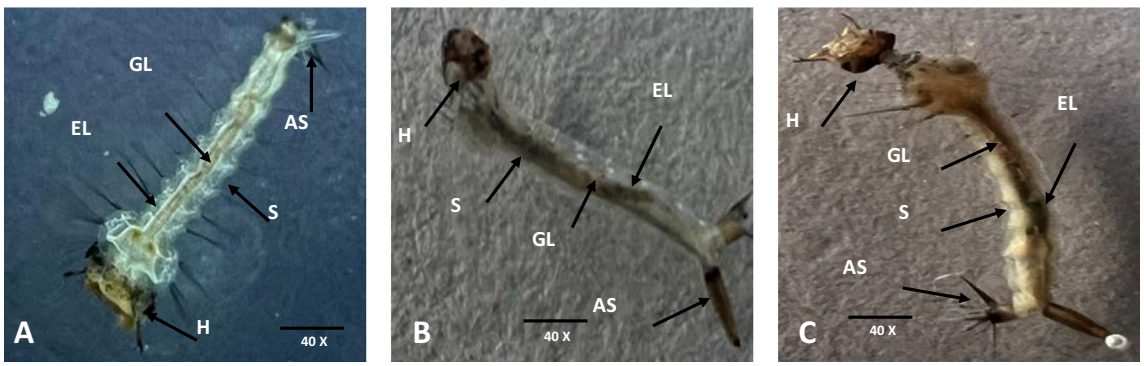


Fig. 3 Photomicrograph showing the abnormality of 4th instar *Cx. quinquefasciatus* larvae treated with essential oils after 24 h. **A** Control untreated larvae. **B** Citriodora, alimentary canal brown. **C** Spearmint gut necrosis. H, head; EL, epithelial layer; GL, gut lumen; S, segments; AS, anal segments

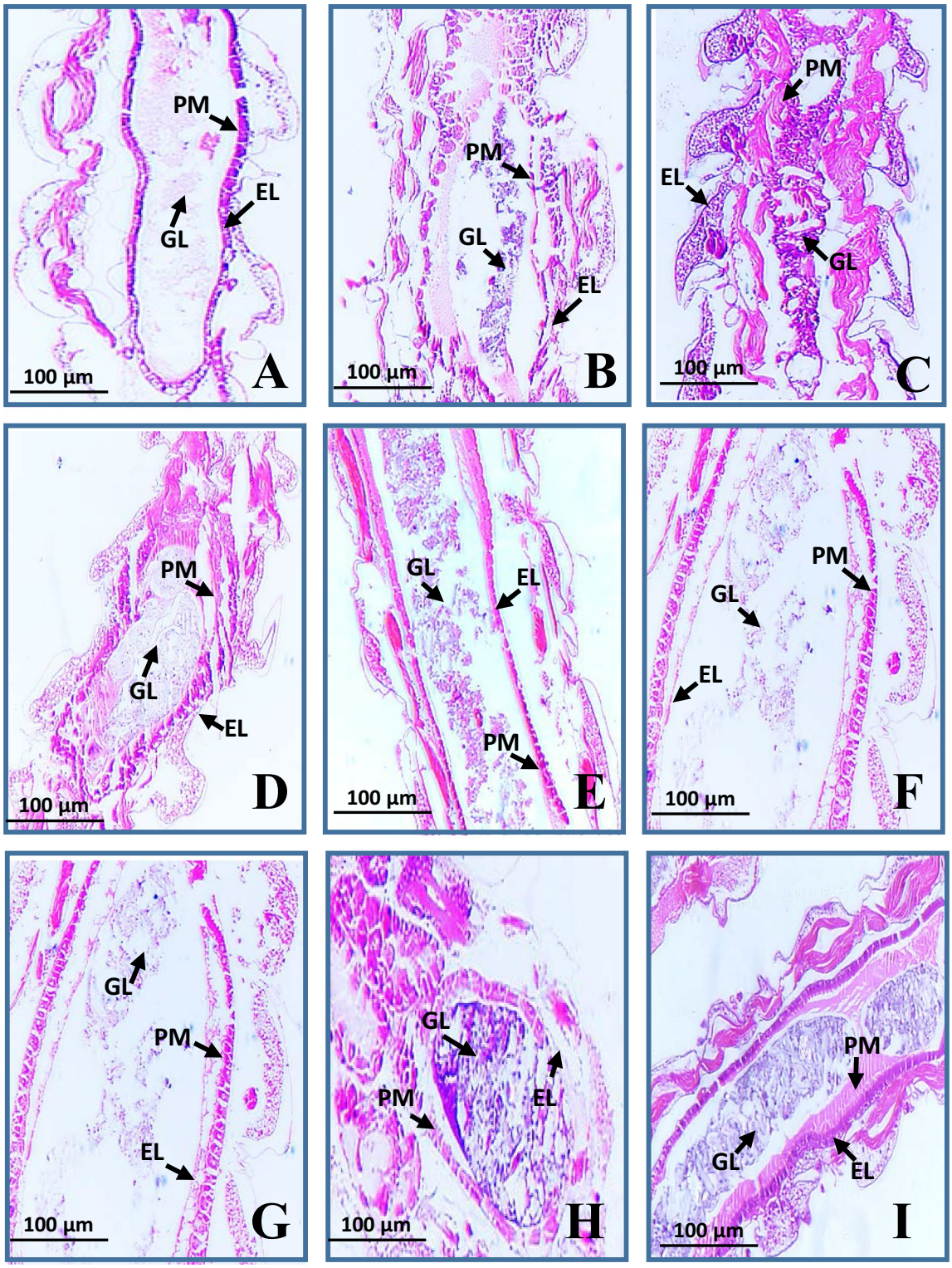


Fig. 4 Lateral section (LS) of mid gut of fourth instar of *Cx. quinquefasciatus* larvae treated with Citriodora oil (CO). **A** Four hours after treatment. **B** Eight hours after treatment. **C** Sixteen hours after treatment. **D** Twenty-four hours after treatment. **E** Larvae treated with Spearmint oil 4 h after treatment. **F** Spearmint oil 8 h after treatment. **G** Spearmint oil 16 h after treatment. **H** Spearmint oil 24 h of treatment. **I** Untreated control. PM, peritrophic membrane; GL, gut lumen; EL, epithelial layer

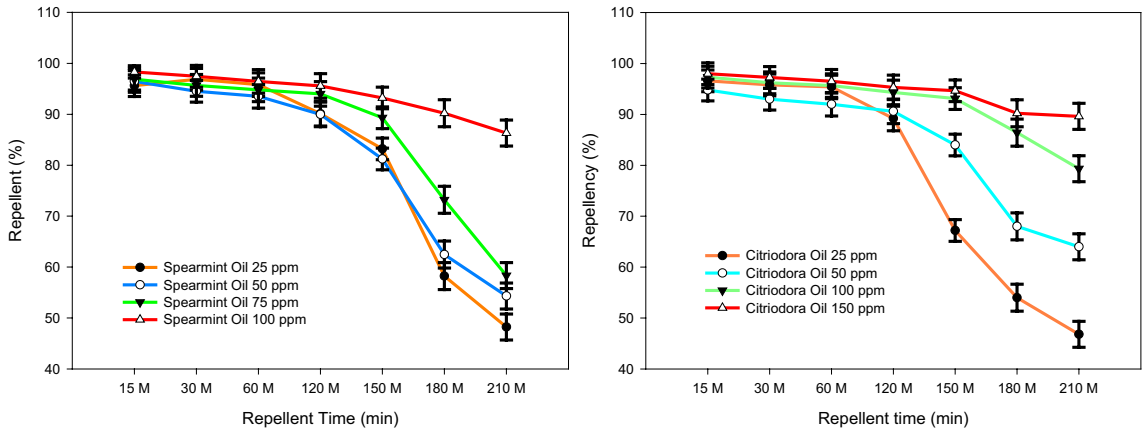


Fig. 5 Repellency of **A** Spearmint and **B** Citriodora essential oil against the mosquito adult *Cx. quinquefasciatus* whereas vertical bars indicate standard error (±SEM)

(97.3%, $F_{4,20} = 18.93$, $P \leq 0.001$) until the maximum protection time of 210 min (Fig. 5B).

Non-target toxicity

BeeTox (in silico) screening of EOs

The in silico toxicological screening of the major metabolites of Spearmint and Citriodora EOs showed that all compounds were non-toxic against the honey bee *Apis mellifera* under acute contact toxicity. Despite this, all compounds (except isomenthol, eucalyptol, 3-hydroxypropanenitrile, 5-(1-bromo-1-methyl-ethyl)-2-methyl-cyclohexanone, and bicyclononadiene diepoxide) induced acute oral toxicity against the bees with a maximum LD_{50} from caryophyllene oxide (194.8894 µg/bee), isomenthone (100.7472 µg/bee), and paramethasone (95.2246 µg/bee), respectively (Table 4).

Aquatic predator (in vitro) toxicity screening

The aquatic mosquito predator (*Tx. splendens*) toxicity test showed that the maximum treatment dosage of temephos (1 ppm) caused the highest morality rate in the aquatic predator (75.4%) and it is significant on other dosages of EOs and the control ($F_{4,20} = 14.73$, $P \leq 0.001$). The highest treatment dosage (200 ppm) of the EOs, however, caused minimal toxicity in the mosquito predator (Spearmint oil (17.2%) and Citriodora oil (15.2%)), respectively) (Fig. 6).

Discussion

Botanical compounds displayed higher ovicidal, larvicidal, and repellent actions towards the initial or adult phases of the arthropod vectors, disturbing respiratory, endocrine, water balance, and nervous systems (Benelli and Cornara 2021; Şengül Demirak and Canpolat 2022). Alkaloids, aromatic chemicals, and EOs derived from herbs are frequently utilized for botanical-based natural repellents (Pavela and Benelli 2016). Traditionally, EOs have been competently utilized against diverse medically challenging insects and crop pests across nations (Sedaghat et al. 2011; Sánchez-Gómez et al. 2022). Additionally, they are possible replacements for synthetic chemicals generally utilized on mosquitoes. EOs are intricate natural blends of phyto-compounds that contain volatile molecules, which are usually terpenes and sesquiterpenes (hydrocarbons), phenylpropenes, and oxygenated hydrocarbons, in different ranges (Moemenbellah-Fard et al. 2020; Noorpisheh Ghadimi et al. 2020; Osanloo et al. 2020). With the ever-growing interest in the use of EOs as an alternative for successful vector control, the present study is aimed at understanding and drawing a meaningful comparison between the impact of different EOs on mosquito larvicidal action, their toxicity, and their effect on the histological profile of the hindgut of *Cx. quinquefasciatus* larvae. Based on the LC_{50} values of the 24-h and 48-h treatments, of the different oils studied, Spearmint oil showed an LC_{50} value of 51.38 ppm during the 24-h exposure while that of Citriodora was 45.36 ppm. Additionally, in the 24-h treatment, Citriodora showed mortality rates of 68% and 74% at 50 and 100 ppm, respectively, while Spearmint oil showed the least mortality (16%) at a 100 ppm concentration.

To select an efficient EO, two criteria should be met: (i) the EOs need to deliver significant mortality rates across the standard larvicidal examinations (WHO 2009) to attain a lethal concentration (LC_{50}) ≤ 100 ppm, and (ii) phyto-chemicals and their peak area percentages should be screened (Pavela 2015). Based on these criteria, all EOs tested showed good larvicidal potential with a LC_{50} less than 100 ppm. Comparable studies have been conducted by Chaiphongpachara et al. (2020) in screening seven marketable herbal oils (East Indian lemongrass, cassia, bay, cinnamon, holy basil, ginger, and sweet basil) for their larvicidal actions on the *Ae. aegypti* dengue larvae.

Table 4 Toxicity of essential oils (Spearmint and Citriodora) ligands against honey bee *Apis mellifera* using BeeTox free online server. The color-coded maps indicate the fragment contributions to toxicity adapted from (BeeTox AI: http://beetoxai.labmol.com.br/)

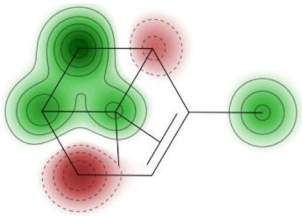
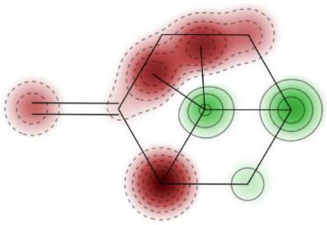
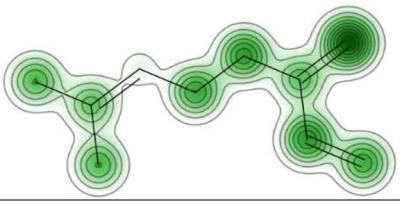
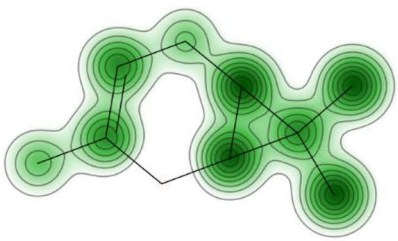
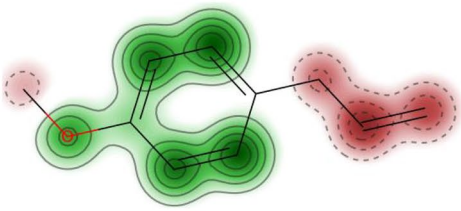
Ligand name	Acute oral Toxicity (LD_{50})	Acute contact toxicity	Contribution Mapping
		Spearmint	
α-pinene	8.6581 µg/bee	Non-toxic (60%)	
β-Pinene	12.9874 µg/bee	Non-toxic (61%)	
β-myrcene	3.7208 µg/bee	Non-toxic (70%)	
3-carene	14.522 µg/bee	Non-toxic (65%)	
Estragole	5.5982 µg/bee	Non-toxic (72%)	

Table 4 (continued)

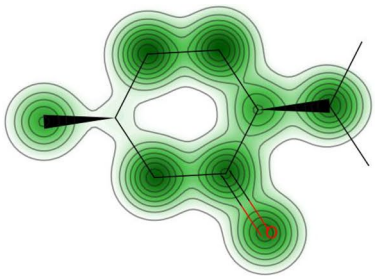
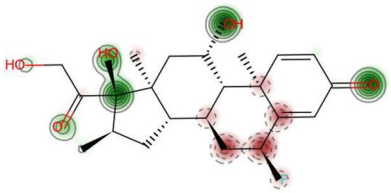
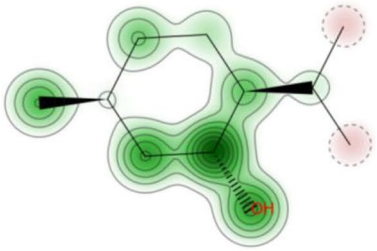
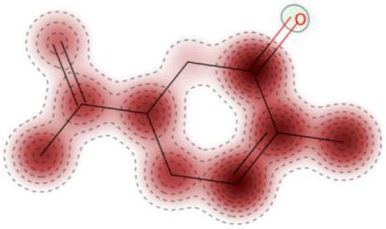
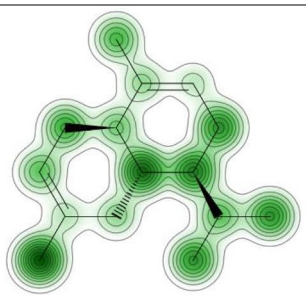
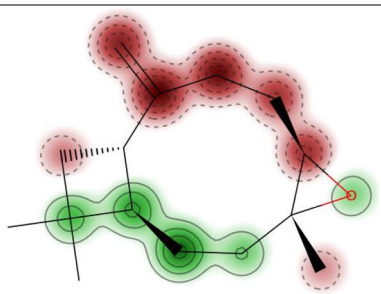
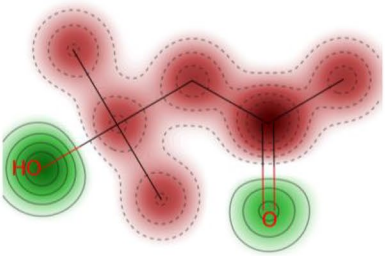
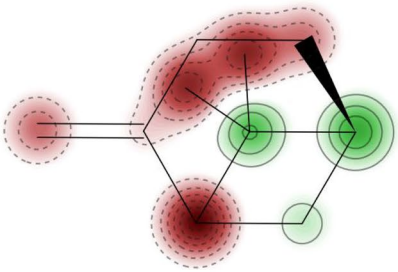
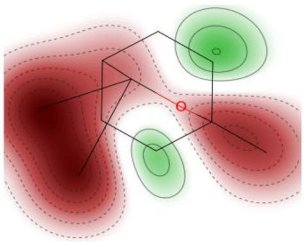
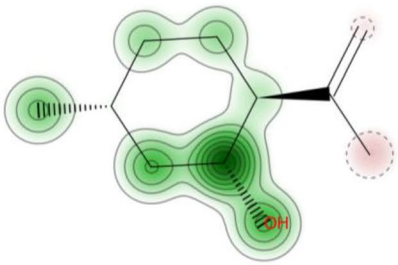
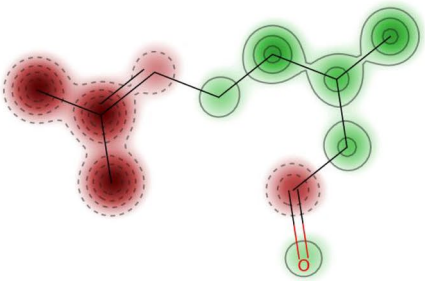
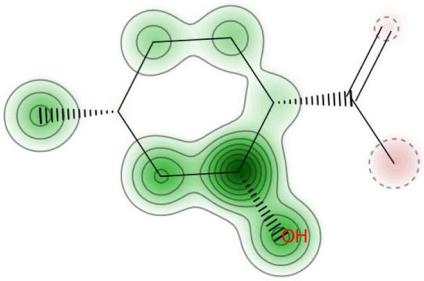
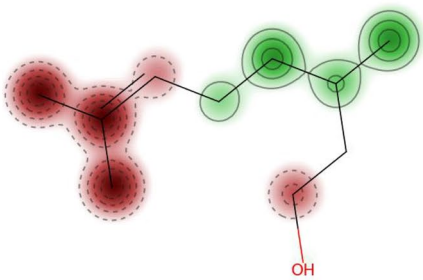
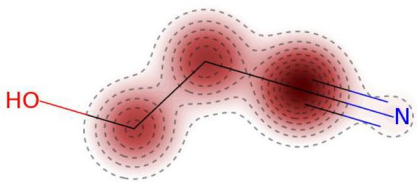
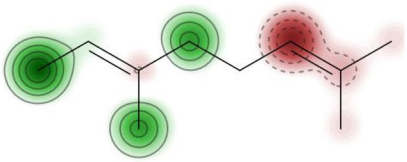
Isomenthone	100.7472 µg/bee	Non-toxic (86%)	
Paramenthasone	95.2246 µg/bee	Non-toxic (82%)	
Isomenthol	-	Non-toxic (88%)	
Carvone	11.6566 µg/bee	Non-toxic (58%)	
δ-cadinene	12.9268 µg/bee	Non-toxic (71%)	
Caryophyllene oxide	194.8894 µg/bee	Non-toxic (73%)	

Table 4 (continued)

		Citriodora	
2-Pentanone, 4-hydroxy-4-methyl-	12.8657 µg/bee	Non-toxic (76%)	
(5S)-6,6-dimethyl-2-methylidenebicyclo[3.1.1] heptane	12.9874 µg/bee	Non-toxic (61%)	
Eucalyptol	-	Non-toxic (70%)	
Isopulegol	5.4564 µg/bee	Non-toxic (86%)	

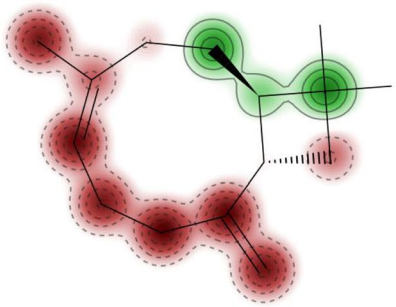
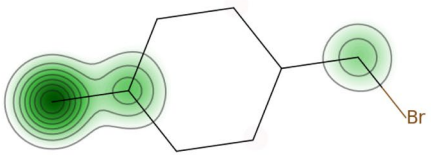
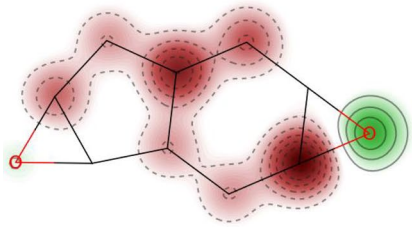
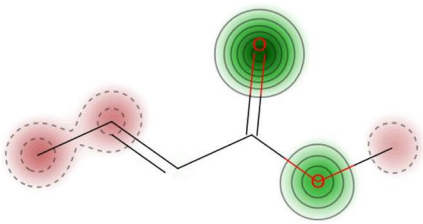
The results showed that cinnamon oil induced significant larvicidal actions with an LC_{50} (0.03 ppm) and LC_{90} (0.04 ppm). The mortality increased with the concentration, which was also observed in our study where the mortality was dose-dependent. When the treated larvae were visible for 48 h in our study, the EOs tested showed low LC_{50} values (less than 50 ppm), indicating progressive toxicity throughout exposure. Interestingly, when the LC_{90} values were compared, Spearmint and Citriodora oils showed LC_{90} values of 68.68 and 50.61 ppm, respectively, in a 24-h exposure

Table 4 (continued)

Citronellal	15.301 µg/bee	Non-toxic (69%)	
(1R,2R,5R)-5-methyl -2-(prop-1-en-2-yl) cyclohexanol	5.4564 µg/bee	Non-toxic (86%)	
Citronellol	4.8696 µg/bee	Non-toxic (65%)	
3-Hydroxypropane nitrile	-	Non-toxic (65%)	
2,6 Octadiene 2,6 dimethyl	7.5822 µg/bee	Non-toxic (63%)	

period, indicating highly active EOs against mosquito larvae (Pavela 2009). The LC_{90} value is also considered important, which most researchers have failed to recognize even though it indicates the dosage that delivered the determined decline towards the ensuing generations of mosquitoes. When we compare the LC_{90} values between the two EOs, though the

Table 4 (continued)

Caryophyllene	19.3759 µg/bee	Non-toxic (64%)	
5-(1-bromo-1-methyl -ethyl)-2 methyl cyclo hexanone	-	Non-toxic (78%)	
Bicyclononadiene diepoxide	-	Non-toxic (72%)	
Crotonic acid methyl ester	4.4691 µg/bee	Non-toxic (71%)	

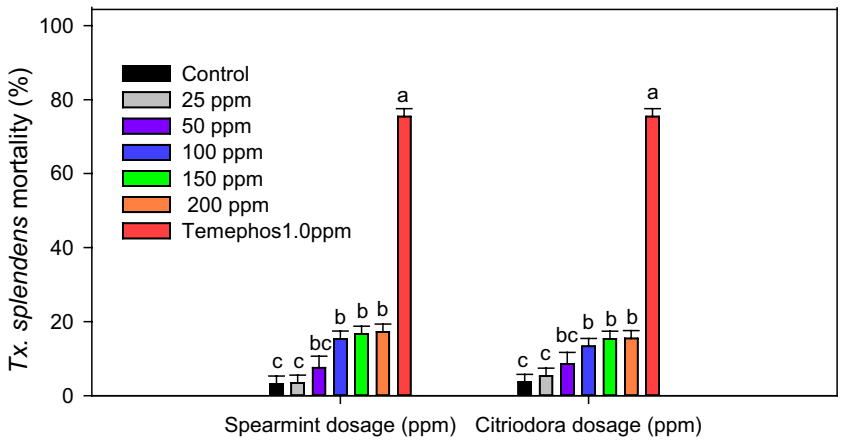


Fig. 6 Non-target toxicity of spearmint and citriodora essential oils against non-target predator *Tx. splendens*. Means (± SEM) followed by the same letters above bars indicate no significant difference ($P \leq 0.05$) by using Probit analysis

24-h LC_{50} values are quite close for the Citriodora (45.36) and Spearmint (51 ppm) oils, the LC_{90} values are remarkably different (146.51 ppm for Spearmint oil and 214.5 ppm for Citriodora oil). This indicates that Spearmint oil has a greater potential as a larvicide. It is well-proven that larvicidal efficacy, a minimal LC_{50} value that does not always require minimal concentration, is adequate to induce mortality. Likewise, this is true in our case for all EOs, which is in agreement with other studies where the estimated LC_{50} and LC_{90} values for EOs derived from *Thymus vulgare* and *Satureja hortensis* for the *Culex* larvae were not the same (de Morais et al. 2007). In addition, Rattan (2010) revealed that the LC_{50} (36 ppm) and LC_{90} (47 ppm) dosages were determined for *Ae. aegypti* larvae post-treatment with *Piper permucronatum*-derived volatile oils. Also, the LC_{50} and LC_{90} values of the EO derived from *Cinnamomum osmophloeum* were 36 ppm and 79 ppm, respectively, when used against the mosquito vector (Cheng et al. 2004). Similarly, the dosages required to attain larval mortality in the EO can be determined by diverse aspects including the selection of the larval instar, cuticle penetration ability of the selected compounds, and ambient temperature, along with the mechanistic actions (Tripathi et al. 2003, 2009; Pavela et al. 2009; Rattan 2010). These underlying factors could explain the difference in mortality of the larvae.

The bioactivity of the EOs towards the arthropod mosquito larvae is related to their chemical composition. In our study, the major constituents of the selected EOs were estragole and carvone for Spearmint oil and citronellal for Citriodora oil. Carvone is reported to possess larvicidal action, which is similar to the Spearmint oil action in our study. These chemicals have also been reported to have insecticidal properties (Santos et al. 2011; Govindarajan et al. 2012; Bullangpoti et al. 2018). The *Eucalyptus* species, which belongs to the Myrtaceae family, is a prevalent harvesting plant in South Asian countries including India, South Korea, and Vietnam. Amid the different *Eucalyptus* species, *Eucalyptus citriodora*-derived EOs are enriched with a higher percentage of the bio-active molecule citronellal. Similarly, our phyto-chemical screening of *E. citriodora* oil also revealed a higher peak area percentage in citronellal (7.47%), citronellol (4.11%), dl-isopulegol (40.42%), and isopulegol (12.14%). Recent studies by Kweka et al. (2016) reported the mosquito larvicidal activity of carvone. Earlier studies by Rahuman et al. (2008) and Nasir et al. (2015) have reported the mosquito larvicidal activity of (−)-isopulegol and other monoterpenes against *An. gambiae*. Additionally, the mosquito larvicidal potential of β-sitosterol was illustrated by Ryan and Byrne (1988). The two monoterpenes, carvone and (−)-isopulegol, are among the chemical constituents identified by GC–MS analysis in Spearmint and Citriodora oils and they have contributed towards the larvicidal activity of these EOs. While the insecticidal activity is attributable to the chemical constituents of the EO tested, in most cases, as EOs are complex mixtures of several compounds, the mechanism that causes this activity against immature larvae is often difficult to identify because the biological effects are due to the individual components acting as synergic mixtures of these components (Maggi and Benelli 2018). Previous screenings in the literature mostly concluded that CVOs were more active against the insect pests as compared to their isolated phyto-compounds. Phenylpropanoids and monoterpene hydrocarbons, recognized as the significant compound classes, induced higher larval mortality. In our study, carvone and estragole, which are present in Spearmint oil, and citronellal and isopulegol, present in Citriodora oil, showed good larvicidal activity with an LC_{50} less than or equal to 50 ppm, thus confirming the constituents responsible for larvicidal performance. It is hypothesized that (Enan 2001; Moola et al. 2023) the lipophilicity of the components plays an important role in larvicidal action, which enhances the passage through the cuticle of the insect, thereby generating toxicity as evidenced by the greater activity of these EOs in our present study. When mosquitoes are exposed to EOs or their constituents, the compounds can disrupt their normal nervous system function. This disruption can lead to hyperactivity, causing the affected insects to exhibit abnormal and erratic behaviors (Patrick et al. 2006).

Histopathological observations on the larvae treated with the EOs tested showed greater damage to mid-gut cells that was progressive over different exposure periods (4-, 8-, 16-, and 24-h treatments) compared to no damage in the control. In all EOs tested, the damage to the peritrophic membrane, epithelial membrane, and foregut, midgut, and hind-gut basement membrane regions was well-illustrated. The structural breakdown resulted in gut lumen leakage and led to major functional malformations. These were mainly due to the volatile constituents present in the EOs such as carvone in Spearmint, and citronellal and dl-isopulegol in Citriodora. Moreover, our results are similar to those of previous reports (Thanigaivel et al. 2018; Chellappandian et al. 2019; Karthi et al. 2020). The degenerative effect of the midgut cells in all Spearmint- and Citriodora-treated larvae seems to agree with the study of intense degenerative reactions in the anterior, posterior, and thorax midgut regions of *Ae. aegypti* larvae caused by targeting ion transporting cells in the gastric caeca of the thorax region and the posterior and anterior midgut of the epithelial cells where osmoregulation connected machineries including H + V-ATPase are significantly expressed in the dengue mosquito larvae (Volkman and Peters 1989). Overall, the essential components (mainly terpenes and sesquiterpenes) act against larvae in modulating the detoxification enzyme coupled with a cytotoxic effect in the immature stages, bringing about greater susceptibility to Spearmint and Citriodora EOs.

By examining various biological endpoints, including different species and life stages, non-target toxicity screening provides a more comprehensive understanding of the potential risks associated with a chemical (Vasantha-Srinivasan et al. 2016; Ponsankar et al. 2016). Non-target toxicity screening helps assess the impact of chemicals on ecosystems, including aquatic and terrestrial environments. This is crucial for protecting biodiversity and maintaining the health of natural ecosystems (Pisa et al. 2015; Stenrod et al. 2016). Our present screening of Spearmint and Citriodora EOs against the non-target mosquito predator had less impact or harmless actions as compared to that of the commercial chemical. This information can be used for understanding the non-target effects of botanical oils and allows for the development of appropriate risk mitigation measures to minimize environmental impact. Moreover, in silico toxicity predictions enable the early identification of potentially hazardous compounds, allowing researchers and regulators to prioritize chemicals for further investigation or regulatory scrutiny (Zulkifli et al. 2023). The present in silico predictions of the major metabolites of Spearmint and Citriodora using the BeeTox server offer a valuable and efficient approach to assessing the potential non-toxic impact of phyto-chemicals, thus contributing to advancements in safety assessment, regulatory compliance, and ethical considerations in scientific research and product development.

Conclusions

The two EOs studied for their larvicidal effects indicated that Spearmint oil is more toxic to larval stages due to its larvicidal action as well as its considerable mortality rate when the larvae are exposed for longer periods (48 h). In search of alternative strategies for mosquito larval control, the use of plant-based EOs like Spearmint and Citriodora oils, along with integrated mosquito management strategies, will provide a sustainable solution. Moreover, the EOs delivered minimal or less toxicity against the aquatic predator *Tx. splendens* (in vitro) and honey bees (in silico). Moving forward, exploring the mechanisms of action of these EOs and their individual components can provide valuable insights. Furthermore, investigating the toxicity of individual chemicals within the EOs and their impact on key detoxifying enzymes in dengue larvae can deepen our understanding of the intricate interactions involved in mosquito control. This avenue of research holds significant promise for the development of targeted and efficient mosquito control strategies. Overall, the study highlights the potential use of natural commercial oils as effective larvicidal agents and emphasizes the importance of integrating plant-based EOs into mosquito management practices.

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Author contribution Pavana Sivadasan Unni: conceptualization of the study, investigation, interpretation of results, validation, writing-original draft, supervision. Pandiyan Kirupaanntha-Rajan: conceptualization of the study, investigation, interpretation of results, validation, writing-original draft, supervision. Prabhakaran Vasantha-Srinivasan: conceptualization of the study, development of methodology, investigation, interpretation of results, validation, writing-review, supervision, project administration, resources. Sridhar Srinivasan: conceptualization of the study, investigation, interpretation of results, writing-review, supervision. Yeon Soo Han: conceptualization of the study, development of methodology, investigation, interpretation of results, writing-review, project administration, funding acquisition. Sengodan Karthi: conceptualization of the study, development of methodology, investigation, interpretation of results, validation, writing-review. Narayanaswamy Radhakrishnan: conceptualization of the study, development of methodology, investigation, interpretation of results, validation, writing-review, resources. Ki Beom Park: conceptualization of the study, investigation, interpretation of results, validation, writing-review, resources. Rajakrishnan Rajagopal: conceptualization of the study, development of methodology, investigation, interpretation of results, validation, writing-review, resources. Sengottayan Senthil-Nathan: conceptualization of the study, development of methodology, investigation, interpretation of results, writing-review, project administration.

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Declarations

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