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Essential oil and fenchone extracted from Tetradenia riparia (Hochstetter.) Codd (Lamiaceae) induce oxidative stress in Culex quinquefasciatus larvae (Diptera: Culicidae) without causing lethal effects on non-target animals

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Abstract

We investigated the larvicidal activity of the essential oil (EO) from *Tetradenia riparia* and its majority compound fenchone for controlling *Culex quinquefasciatus* larvae, focusing in reactive oxygen and nitrogen species (RONS), catalase (CAT), glutathione S-transferase (GST), acetylcholinesterase (AChE) activities, and total thiol content as oxidative stress indicators. Moreover, the lethal effect of EO and fenchone was evaluated against *Anisops bouvieri*, *Diplonychus indicus*, *Danio rerio*, and *Paracheirodon axelrodi*. The EO and fenchone (5 to 25 µg/mL) showed larvicidal activity (LC_{50} from 16.05 to 18.94 µg/mL), followed by an overproduction of RONS, and changes in the activity of CAT, GST, AChE and total thiol content. The Kaplan-Meier followed by Log-rank (Mantel-Cox) analyses showed a 100% survival rate for *A. bouvieri*, *D. indicus*, *D. rerio*, and P. axelrodi when exposed to EO and fenchone (262.6 and 302.60 µg/mL), while *α*-cypermethrin (0.25 µg/mL) was extremely toxic to these non-target animals, causing 100% of death. These findings emphasize that the EO from *T. riparia* and fenchone serve as suitable larvicides for controlling *C. quinquefasciatus* larvae, without imposing lethal effects on the non-target animals investigated.

INTRODUCTION

Lymphatic filariasis (LF), also known as elephantiasis, is a mosquito-borne and neglected disease caused mainly by the nematode *Wuchereria bancrofti* (Filariodidea) (World Health Organization 2023a), which obstructs the lymphatic flow in the human organism, resulting in abnormal accumulation of fluids, particularly in the lower limbs with thickening of the skin, and underlying tissues (Matapo et al. 2023). The manifestations also include the enlargement of the testicles, and excessive growth or swelling of the limbs, breasts, and scrotal sac (Tamadaho et al. 2023). These factors have elevated LF to one of the diseases with the highest rates of permanente or long-term disability worldwide (World Health Organization 2023a).

In fact, according to World Health Organization (2023a), in 2021, over 882.5 million people worldwide remained at risk of LF infection, including up to 25 million men with hydrocele, 15 million people with lymphoedema, and at least 36 million people remain with other manifestations. Currently, 120 million people worldwide are infected, and among them, 40 million suffer from disabilities or disfigurement, particularly in Asia, Africa, Western Pacific, Caribbean and America (World Health Organization 2023b). In the Americas, about 12.6 million of cases were registered, with 90% occurring in Haiti, which, along with Brazil, Guyana, and the Dominican Republic, are recognized as endemic countries (Pan American Health Organization 2023). In these regions, *W. bancrofti* is transmitted by mosquitoes from the genus *Culex*, including *C. quinquefasciatus* (Say, 1823), *C. pipiens* (Linnaeus, 1758), *C. tritaeniorhynchus* (Giles, 1901), *C. antennatus* (Becker, 1903), *C. thelerei* (Theobald, 1903), and *C. perexiguus* (Theobald, 1903) (Diptera: Culicidae) (de Castro Oliveira et al. 2022; Mohamed et al. 2023). In Brazil, the locations where females *C. quinquefasciatus*, main vector of LF, lay their eggs, and their larvae develop into adult insects, are generally, collection of stagnant water polluted with decomposing organic matter, mainly untreated domestic wastewater discharged into the environment (Brazil Ministry Health 2023), especially in urban

areas lacking sanitation, allowing breeding sites near homes, that despite feeding on the blood animals, this vector is highly anthropophilic, with preference for human blood (Brazil Ministry Health 2023).

Currently, studies have demonstrated that the use of botanical larvicidal, particularly essential oils (EOs), and their compounds with several mechanism of action for controlling mosquitoes larvae, is an important strategy in preventing mosquito-borne diseases (Budiman et al. 2021; Subaharan et al. 2021). EOs, and their compounds obtained generally by hydrodistillation, and other isolation methods, respectively, of various plants parts, exhibited various mechanisms of action, including inhibition of acetylcholinesterase (AChE) (Shahriari et al. 2018), oxidative stress, apoptose and necrose cell (Costa et al. 2022), inhibition of catalase, glutathione-S-transferase (Johnson et al. 2021), mitocondrial, and DNA damage (Kanis et al. 2018).

Another advantage of using EOs, and their compounds is their lower toxicity against non-target animals when compared with synthetic chemical larvicides as organochlorines, carbamates, and pyrethroids, still utilized in some countries (Koodalingam and Dayanidhi 2021; Ganesan et al. 2023). For exemple, lambda-cyhalothrin, thiamethoxam, *α*-cypermethrin, temephos, among other synthetic larvicides, were extremely toxic against non-target animals of the genera *Cyprinus* (Cyprinidae), *Diplonychus* (Heteroptera), *Toxorhynchites* (Culicidae), *Anisops* (Hemiptera), and *Gambusia* (Poeciliidae) (LC_{50} ranging from 0.22 to 5.82 µg/mL) (Clasen et al. 2018; de Oliveira et al. 2022a, b). In contrast, EOs obtained of plants of the genera *Syzygium* (Myrtaceae), *Tetradenia* (Lamiaceae), *Piper* (Piperaceae), *Ichnocarpus* (Apocyanaceae), as well as the compounds *α*-humulene, *β*-elemene, *β*-caryophyllene, germacrene D, and fenchone, no showed significant toxicity against these animals (LC_{50} ranging from 189.24 to 20374.26 µg/mL) (Govindarajan and Benelli 2016; Govindarajan et al. 2016; de Oliveira et al. 2022a, b).

Tetradenia riparia (Hochstetter.) Codd (Lamiaceae), commonly known as false myrrh, lavandula, and pau-de-incenso in various countries of Africa and South America, is a shrub characterized with white flowers, dense leaves, and a pleasant aroma (de Oliveira et al. 2022b). Studies performed with EOs, extracts or compounds isolated of *T. riparia*, such as 9β,13β-epoxy-7-abietene, *α*-pyrone, 6,7-dehydroroyleanone, and fenchone demonstrated significant biological activities, including antibacterial (Gazim et al. 2010), antmalarial (Campbell et al. 1997), anticholinesterase (Amoo et al. 2012), repellency (Omolo et al. 2004), antileishmania (Cardoso et al. 2015), antioxidant (Gazim et al. 2014), and anti-mycobacterium (Baldin et al. 2018). Additionally, the EO, and fenchone exhibited acaricidal activity against *Rhipicephalus* (Boophilus) *microplus* (Ixodidae) (Gazim et al. 2011), as well as activity against *Aedes*, and *Anopheles* larvae (Fernandez et al. 2014; de Oliveira et al. 2022b).

In this context, given the noteworthy larvicidal activity exhibited by EO from *T. riparia*, and fenchone against Culicidae larvae, and considering the importance of *C. quinquefasciatus* in the transmission of LF, mainly in Brazil, this study aimed to investigate the EO from *T. riparia*, and its majority compound fenchone as eco-friendly larvicides for the control of *C. quinquefasciatus* larvae.

MATERIAL AND METHODS

Chemicals and reagents

Phosphate buffer solution (pH 7.3), potassium phosphate buffer (0.1 M, pH 7.4), bovine serum albumin (pH 7, ≥ 98%) (BSA), hydrogen peroxide (H_2O_2) (≥ 35%), 1-chloro-2,4-dinitrobenzene (CDNB) (≥ 99%), Ethylenediaminetetraacetic acid (EDTA) (≥ 98%), Glutathione (GSH) (analytical standard), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (≥ 99%), acetylthiocholine iodide (AChI) (≥ 99%), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (≥ 95%), 2',7'-dichlorofluorescein (DCF) (≥ 90%), anhydrous sodium sulfate (Na_2SO_4), *n*-alkane series (C_8–C_{30}) (Supelco), dimethyl sulfoxide (DMSO) (≥ 99%), *α*-cypermethrin (PESTANAL®, analytical standard), hexane (HPLC) (≥ 99%), ethyl acetate (HPLC) (99.9%), and methanol (HPLC) (99.9%) were purchased from Merck (Brazil). Fenchone (≥ 98%) was purchased from Boc Sciences, (New York, USA).

Collecting and identification of T. riparia

The *T. riparia* plant was collected in Manaus, Amazonas, Brazil (3◦4'24.2726"S and 59◦57'41.299"W) in January 2022. The collecting was authorized by Sistema de Autorização e Informação em Biodiversidade (SISBIO) (No. 78390-1), and registered in the Sistema Nacional de Gestão do Patrimonio Genético e do Conhecimento Tradicional Associado (SisGen) (No. A563032). The sample was identified by botanist Maria Rosalva da Costa Bilby. The voucher No. 010868 was deposited in the Herbarium of the Universidade Federal do Amazonas.

Prior to extraction of the EO, *T. riparia* leaves were carefully washed with tap water, dried in the absent of sunlight, ground into powders, and stored in plastic recipient for subsequent processing (de Oliveira et al. 2022b).

Extraction of the essential oil

To extraction of the EO, about 200 g of powdered *T. riparia* leaves were subjected to hydrodistillation using a Clevenger-type apparatus (de Oliveira et al. 2022b). The resulting EO was collected, dried using anhydrous sodium sulfate (Na_2SO_4), and stored in amber glass at − 4°C for subsequent analysis. This procedure was performed in triplicate for a duration of 3 h. The EO yield was determined using the following equation: EO (%) = Weight of EO / Weight of samples x 100.

GC-MS and GC-FID analyses

Prior to injection, 1 mg of the EO was diluted in 1 mL of ethyl acetate. Then, 1 µL was used for qualitative and quantitative analyses. For the GC-MS analyses were used a Trace GC Ultra model coupled to the ISQ mass spectrometer (Thermo Scientific) with a capillary column TR-5 (30 m × 0.25 mm × 0.25 µm) while the GC-FID analyses were performed on an Agilent G-1860 Plus model with capillary column HP-5 (30 m × 0.32 mm × 0.25 µm). The Xcalibur software v.2.2 controlled, and processed the data acquisition. The conditions of operating were reported in our study (de Oliveira et al. 2020).

The retention indexes (RI) were obtained using the *n*-alkane (C_8-C_{30}) and the equation of (van Den Dool and Dec. Kratz 1963). The identification of each compound was done by comparison of the obtained RI with the ones found in the literature (de Oliveira et al. 2022b); Adams 2007; NIST 2020; in-house library).

C. quinquefasciatus rearing

The collection of *C. quinquefasciatus* larvae was conducted in the lake at the Centro de Estudos dos Quelônios da Amazônia localized at the Instituto Nacional de Pesquisas da Amazônia (INPA), using an entomological shell. The larvae were transferred to a recipient (4 L) containing 2 L of distilled water, and acclimated under controlled conditions of relative humidity (80 ± 5%), and temperature (28 ± 2 ◦C) for 24 h. Then, the larvae were identified using a taxonomic key Consoli and Oliveira 1994; Brazil Health Ministry (2011). The procedures for larvae rearing were performed following the Manual for breeding Diptera of veterinary importance: *Culex quinquefasciatus* and *Aedes aegypti*, as described by Empresa Brasileira de Pesquisa Agropecuária (2023).

Larvicidal activity

The evaluation of the larvicidal activity of the EO from *T. riparia*, and fenchone against *C. quinquefasciatus* larvae was carried out in accordance with the Guidelines for Laboratory and Field Testing of Mosquito Larvicides from World Health Organization (World Health Organization 2005), under controlled conditions of relative humidity (80 ± 5%), and temperature (28 ± 2 ◦C). Briefly, *C. quinquefasciatus* larvae (*n* = 200) of 3rd instar were transferred for five recipients (200 mL) containing 99 mL of distilled water, and concentrations ranging from 5 to 25 µg/mL of the EO from *T. riparia*, and fenchone prepared in 1 mL of DMSO. Additionally, a concentration of 0.25 µg/mL *α*-cypermethrin was used as positive control, as previously described by (Subahar et al. 2022). DMSO at 1% was used as negative control. The assay was conducted in five replicates. Following a 24-hour exposure period, the percentage of larvicidal activity for each concentration was determined using the equation: Larvicidal activity (%) = Total of dead larvae / Total of treated larvae x 100.

Toxicity assay in non-target animals

The lethal effect of the EO from *T. riparia*, and fenchone was carried out in accordance with the methodology reported (Sivagnaname and Kalyanasundaram 2004), with slight modifications. The assays were conducted on adult specimes of *Anisops bouvieri, Diplonychus indicus, Danio rerio, and Paracheirodon axelrodi*, which are recognized as predators of Culicidae larvae (Clasen et al. 2018; de Oliveira et al. 2022a). *A. bouvieri*, and *D. indicus* were also collected from the same natural habitat of *C. quinquefasciatus* larvae, while *D. rerio*, and *P. axelrodi* were purchased in local market. After a 48-hour acclimatization period in recipient (90 cm in diameter and 40 cm deep) containing 5 L of water from the breeding habitat, the invertebrates were identified using a taxonomic key (Hamada et al. 2014), and transferred to recipients (1 L) containing 499 mL of water from breeding habitat, and concentrations of 262.6 and 302.60 µg/mL of the EO from *T. riparia*, and fenchone, respectively, which were obtained by multiplying the LC_{90} values by 10. As positive control, was utilized *α*-cypermethrin at 0.25 µg/mL, while

DMSO at 1% was used as negative control. Following a 60-day exposure period, the Kaplan-Meier followed Log-rank (Mantel-Cox) analyses were employed to estimate the survival curve for all animals (Kishore et al. 2010). All assays were carried out with five replicates under controlled conditions of relative humidity (80 ± 5%), and temperature (28 ± 2°C).

Sample preparation for enzymatic assays

At the conclusion of the 24-hour exposure period in larvicidal assay, *C. quinquefasciatus* larvae, which has been treated with 25 µg/mL of the EO from *T. riparia*, and fenchone, 0.25 µg/mL of *α*-cypermethrin, and 1% of DMSO, were homogenised in a phosphate buffer (pH 7.3 at 0.1 M) at a ratio of 1 mg larvae per 10 mL of buffer), and then centrifuged at 4000 rpm for 5 min. The resulting supernatants were transferred to Eppendorf tubes, and stored at − 4°C for subsequent analysis of oxidative stress indicators, including reactive oxygen and nitrogen species (RONS), catalase (CAT), glutathione S-transferase (GST), acetylcholinesterase (AChE), and total thiol content (Abolaji et al. 2015; Janner et al. 2021; Johnson et al. 2021). The protein content was also determined using bovine serum albumin (BSA, 1 mg mL^{-1}) as the standard for quantification (Lowry et al. 1951).

Measurement of reactive oxygen and nitrogen species (RONS)

To assess whether the EO from *T. riparia*, and fenchone could stimulate the overproduction of RONS in *C. quinquefasciatus* larvae, 2',7'-Dichlorofluorescein (DCFH) oxidation was used as an indicator of oxidative stress, following methodology previously reported (Pérez-Severiano et al. 2004), and adapted for larvae homogenate (Abolaji et al. 2015). About 5 µL of each supernatant (1:10 dilution), 150 µL of potassium buffer (pH 7.4 at 0.1 M), 40 µL of distilled water, and 5 µL of DCFH-DA were transferred into a 96-well plate, followed by incubation at 37°C for 60 min. A standard curve was generated using increasing concentrations of DCF, incubated in parallel, resulting in a final concentration of 5 µL. The fluorescence resulting from DCFH oxidation was measured over a 10-min period with 30-seconds intervals, using SpectraMax plate reader (Molecular Devices, USA) with excitation at 488 nm, and emission at 525 nm. As control groups were used *α*-cypermethrin, and DMSO. The rate of DCF formation was expressed in percentage of the control groups. All assays were performed in triplicate for each supernatant.

Catalase activity (CAT)

The CAT activity was performed according to the procedure initially described by (Aebi 1984), with modifications (Abolaji et al. 2015). A solution comprising 1.8 mL of phosphate buffer (pH 7.3, 0.1 M), and 180 µL of H_2O_2 (300 mM) was mixed with 20 µL of each supernatant at 25°C. The reduction of H_2O_2 was monitored over a 2 min with 10 sec intervals, measured at 240 nm using a UV–visible spectrophotometer. The results were expressed as µmol of H_2O_2 consumed/min/mg of protein.

Glutathione S-transferase (GST) activity

To determine GST activity, 1-chloro-2,4-dinitrobenzene (CDNB) was utilized as substrate, and the procedure was carried out as described by Habig (Habig and Jakoby 1981). Briefly, 20 µL of each supernatant was mixed with 270 µL of solution A, comprising 20 µL of phosphate buffer (0.25 M), EDTA (2.5 mM), 10.5 µL of distilled water, and 500 µL of GSH (pH 7.0, 0.1 M) at 25°C, followed by the addition of 10 µL of CDNB (25 mM). Absorbance was read at 340 nm with 10 sec intervals over a 5 min. GST activity was expressed in µmol/min/mg protein.

Acetylcholinesterase (AChE) activity

The AChE activity was carried out according to the colorimetric method described by (Ellman et al. 1961), and adapted for a 96-well plate (Abolaji et al. 2015). In brief, the reaction mixture containing 135 µL of distilled water was supplemented with 20 µL of phosphate buffer (pH 7.3, 100 mM), 20 µL of DTNB (10 mM), 20 µL of AChI (8 mM), and 20 µL of each supernatant. Absorbance was read at 412 nm every 30 sec over a 2 min using a microplate reader (ELx800, Biotek, USA). The AChE activity was expressed as µmol/min/mg protein. All procedures were performed at 25°C under absence of light. All experiments were carried out in triplicate.

Determination of total thiol content

The determination of thiol content was performed in accordance with the method described by Ellman (Ellman 1959), with slight modifications. A total of 20 µL of each supernatant was mixed with Ellman's reagent comprising of 150 µL of potassium phosphate buffer (pH 7.4, 0.1 M), 15 µL of DTNB (1 mM), and 15 µL of distilled water. This mixture was then incubation for 30 min at room temperature. Subsequently, the absorbance was measured at 412 nm, and the data obtained were utilized to calculate the total thiol levels expressed as µmol/mg protein, using GHS as standard.

Statitical analysis

Probit analysis was conducted using IBM® SPSS® Statistics software to estimate the LC_{50}, LC_{90} values, linear equations, Chi-square, and degree of freedom (Giatropoulos et al. 2018). Subsequently, an unpaired *t*-test ($p \leq 0.05$) was employed to assess significant differences in LC_{50}, and LC_{90} values. The Kaplan-Meier followed Log-rank (Mantel-Cox) analyses ($p \leq 0.05$) were applied to construct the survival curve of the non-target animals (Kishore et al. 2010). Furthermore, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$) was use to identify the statistical differences between the treated groups in the biochemical assays, with GraphPad Prism® 9 software (Thakor et al. 2017).

RESULTS AND DISCUSSION

Essential oil analysis

The EO was extracted from *T. riparia* leaves yielding 2.1 ± 0.13%. Analysis by CG-MS, and CG-FID revealed that oxygenated monoterpenes (41.01%) constituted the main chemical class of the EO, followed by oxygenated sesquiterpenes (25.17%), monoterpenes hydrocarbons (21.79%), and sesquiterpenes

hydrocarbons (12.03%). Fenchone (38.00%), an oxygenated monoterpene, was the majority compound. Other compounds such as 14-hydroxy-9-epi-(*E*)-caryophyllene (10.00%), caryophyllene oxide (6.58%), *β*-caryophyllene (7.31%), *α*-pinene (7.40%), camphor (5.31%), camphene (4.64%), and sabinene (4.48%) were also identified in the EO with low percentages (Table 1).

Table 1
Chemical compounds identified in the EO extracted from the leaves of *T. riparia*.

N°	Compounds	RI^{Calc}	RI^{Lit}	Area (%)	Identification
1	*α*-Pinene	929	939	7.40	RI, MS
2	Camphene	950	954	4.64	RI, MS
3	Sabinene	961	975	4.48	RI, MS
6	*β*-Pinene	975	979	2.39	RI, MS
5	Limonene	1024	1029	2.87	RI, MS
6	Fenchone	1086	1096	38.00	RI, MS
7	Fenchol < endo>	1138	1146	1.33	RI, MS
8	Camphor	1140	1147	5.31	RI, MS
9	Borneol	1152	1160	0.85	RI, MS
10	*α*-Gurjunene	1410	1409	1.21	RI, MS
11	*β*-Caryophyllene	1414	1419	7.31	RI, MS
12	*γ*-Elemene	1435	1441	3.51	RI, MS
13	6-epi-shyobunol	1505	1517	0.60	RI, MS
14	Cubebol	1510	1518	0.36	RI, MS
15	(-)-Cubenol	1604	1610	3.15	RI, MS
16	Caryophyllene oxide	1613	1617	6.58	RI, MS
17	14-hydroxy-9-epi-(*E*)-caryophyllene	1665	1669	10.00	RI, MS
	Total identified			100	
	Monoterpenes hydrocarbons			21.79	
	Oxygenated monoterpenes			41.01	
	Sesquiterpenes hydrocarbons			12.03	
	Oxygenated sesquiterpenes			25.17	

RI^{calc} - Retention index calculated using *n*-alkanes (C_8-C_{30}) on the TR5 column. RI^{lit} - Retention index literature (Adams 2007; NIST 2023; in-house library). Relative area calculated from the peak area relative to the total peak area in the GC-FID chromatogram average of three replicates. MS - Mass spectra compared with literature (Adams 2007; NIST 2023; in-house library).

The yield, and chemical profile described above were similar to our previous study, in which we obtained EO with a yield of 1.4 ± 0.17%, and fenchone (38.62%) as majority compound (de Oliveira et al. 2022b). In contrast to our findings, (Gazim et al. 2014), and (Baldin et al. 2018) reported oxygenated sesquiterpenes as dominant compounds, constituting 45.18 to 64.33% of the EO, and 14-hydroxy-9-epi-(*E*)-caryophyllene ranging from 12.70 to 24.36%, as majority compound.

The qualitative, and quantitative differences of compounds in the EOs could be ascribed to environmental factors, as well as the morphological and genetic characteristics of *T. riparia*, which influence the synthesis of secondary metabolites, such as fenchone in some specimens, and isopimara-8,15-diene, viridiflorol, *α*-humulene, 14-hydroxy-9-epi-(*E*)-caryophyllene, and *α*-terpineol in other specimens (Fernandez et al. 2014; Hannweg et al. 2016).

Larvicidal activity

The EO extracted from *T. riparia* leaves, evaluated at concentrations ranging from 5 to 25 µg/mL, exhibited larvicidal activity against *C. quinquefasciatus* larvae, ranging from 1 ± 0 to 90 ± 5%, with LC_{50} of 8.53 µg/mL, and LC_{90} of 9.59 µg/mL. Interestingly, its exhibited higher activity than fenchone, also evaluated in these concentrations, which demonstrated larvicidal activity ranging from 6 ± 2 to 79 ± 7%, with an LC_{50} of 10.39 µg/mL, and LC_{90} of 11.61 µg/mL. Additionally, an unpaired *t*-test revealed significant statistical differences between their respective LC_{50} and LC_{90} values ($t = 2.274$; df = 4; $p < 0.05$) (Table 2). This is the first report describing the larvicidal activity of the EO from *T. riparia* leaves, and its majority compound fenchone against *C. quinquefasciatus* larvae. In Addition, the positive control *α*-cypermethrin at 0.25 µg/mL exhibited 100 ± 0% larvicidal activity. No dead larvae was identified in negative control.

Table 2
Lethal concentrations of the EO from *T. riparia*, and fenchone estimated against *C. quinquefasciatus* larvae.

Product	Concentrations (µg/mL)	Mortality (%)	LC_{50} (µg/mL) (LCL-UCL)	LC_{90} (µg/mL) (LCL-UCL)	χ^2 (Df)*	Linear equation
EO	5	1 ± 0	16.05[a] (15.182–16.947)	26.26[a] (24.178–29.269)	7.779(3)*	Y = -7.227 + 5.995x
	10	10 ± 3				
	15	39 ± 5				
	20	72 ± 4				
	25	90 ± 5				
Fenchone	5	0 ± 0	18.94[b] (17.685–20.025)	30.26[b] (27.585–34.390)	2.565(3)*	Y = −8.052 + 6.303x
	10	6 ± 2				
	15	21 ± 4				
	20	57 ± 5				
	25	79 ± 7				
α-cypermethrin	0.25	100				

LC_{50} and LC_{90} - Concentrations needed to kill 50 and 90% of larvae. LCL - Lower confidence limit of 95%. UCL - Upper confidence limit of 95%. * Since goodness-of-fit test is significant ($p < 0.05$), a heterogeneity factor was used in the calculation of confidence limits (CL). Df - Degree of freedom. Letters (a-b) in the same column indicate statistical difference (Unpaired *t*-test, $p < 0.05$).

Against other Culicidae, the EO from *T. riparia*, and fenchone demonstrates larvicidal activity against *Aedes aegypti*, *A. albopictus* (LC_{50} from 29.31 to 1.590 µg/mL) (Fernandez et al. 2014; Zardeto et al. 2022; de Oliveira et al. 2022b), *Anopheles nuneztovari*, *An. triannulatus*, *An. darlingi*, and *An. albitarsis* (LC_{50} from 30.21 to 35.21 µg/mL) (de Oliveira et al. 2022).

Studies also reported the efficacy of the OE from *T. riparia* as egg-laying inhibitor (90.20%), eggs hatching inhibitor (LC_{50} of 0.114 µg/mL), larvicidal (LC_{50} of 1.222 µg/mL), and adulticidal (LC_{50} of 0.534 µg/mL) against *Rhipicephalus* (Boophilus) *microplus* (Ixodidae) (Gazim et al. 2011). Additionally, other biological activities of the EO this species including activities against *Leishmania amazonensis* (Tripanossomatida) (IC_{50} from 13.31 to 15.66 µg/mL) (Cardoso et al. 2015), *Streptococcus mitis* (IC_{50} of 31.2 µg/mL), *S. mutans* (IC_{50} of 62.5 µg/mL), *S. sobrinus* (Streptococcaceae) (IC_{50} of 31.2 µg/mL), and *Lactobacillus casei* (Lactobacillaceae) (IC_{50} of 62.5 µg/mL) (Fernandez et al. 2014).

Toxicity assay in non-target animals

The Kaplan-Meier followed by Log-rank (Mantel-Cox) analyses, presented in Fig. 1a to 1c, reveals a 100% survival rate for *A. bouvieri* (Chi-square 39.00, df 3, $p < 0.05$), *D. indicus* (Chi-square 39.00, df 3, $p < 0.05$), *D. rerio* (Chi-square 38.00, df 3, $p < 0.05$) and *P. axelrodi* (Chi-square 38.00, df 3, $p < 0.05$) after a 60-day exposure period to concentrations of 262.6 and 302.60 µg/mL of the EO from *T. riparia* and fenchone, respectively, when compared to *α*-cypermethrin at 0.25 µg/mL, that was extremely toxic to these animals, causing 100% of death.

In addition on these findings, we reported the low toxicity of the EO from *T. riparia* (200 to 1000 µg/mL) and fenchone (20 to 100 µg/mL) against other non-target animals, specifically in *Toxorhynchites haemorrhoidalis* (Culicidae), and *Gambusia affinis* (Poeciliidae), with LC_{50} values ranging from 170.50 to 733.79 µg/mL, with suitability indices, and predator safety factor ranging from 13.91 to 31.91 (de Oliveira et al. 2022b). In this context, a review published by Pavela (2015) ratify that botanical larvicides as EOs, and natural compounds applied in lower concentrations may also lead to a significant reduction in the population density of the mosquitoes without exerting negative impact on non-target animals.

Measurement of reactive oxygen and nitrogen species (RONS)

The analysis of DCFH oxidation clearly shows substantial overproduction of RONS, indicating oxidative stress in *C. quinquefasciatus* larvae followed exposure to 25 µg/mL of the EO from *T. riparia*, fenchone, and *α*-cypermethrin, that demonstrated highest production of RONS ($F_{3.8} = 858.5$; $p < 0.05$). Conversely, the lower production of RONS was observed with DMSO at 1% (Fig. 2a). These findings confirm that both botanical and synthetic larvicides trigger the overproduction of RONS. Consistent with literature, this phenomenon leads to oxidative stress on cellular components, disrupting the cellular redox balance and causing an excessive generation of free radicals in mosquito larvae, ultimately resulting in their death (Abolaji et al. 2015; Costa et al. 2022).

Although we did not investigate cell damage explicitly, we propose that the overproduction of RONS, accompanied by oxidative stress were the primary factors that contributing to the death of *C. quinquefasciatus* larvae. In fact, significant oxidative stress biomarkers, such as protein oxidation, lipid peroxidation, accompanied by mitochondrial dysfunction, cell collapse, and cell death in *A. aegypti* larvae were observed after exposure to the extract of *Xylaria* sp. (Xylariaceae) (Costa et al. 2022).

Other study reported substantial vacuolation, a decrease in the activity of tricarboxylic acid cycle enzymes, decreased ATP content in the mitochondrial membrane, interruption of mitochondrial biosynthesis, followed by apoptosis and necrosis, DNA damage, altered membrane permeability and fluidity, and compromised integrity in the midgut cells of *A. aegypti* exposed to *α*-terthienyl, a natural phytotoxin isolated originally from *Flaveria bidentis* (L.) Kuntze (Asteraceae) (Zhang et al. 2019).

In a similar context, *Anopheles stephensi* larvae (Culicidae) exposed to plumbagin, a natural compound isolated of *Plumbago zeylanica* roots (Plumbaginaceae), exhibited significant alterations in AChE, GST, superoxide dismutase, cytochrome P450, *α*, and *β*-esterase, as revealed by biochemical assays in

comparison to control groups ($p < 0.05$), and the subsequent histological examination demonstrated that midgut, hindgut, and muscles were the most affected tissues, with significant changes, including the separation, and collapse of the epithelial layer and cellular vacuolization (Pradeepa et al. 2016).

In *Drosophila melanogaster* (Drosophilidae) exposed to imidacloprid (Janner et al. 2021), 4-vinylcyclohexene 1,2-monoepoxide, and 4-vinylcyclohexene diepoxide (Abolaji et al. 2015), the overproduction of RONS, and oxidative stress induced a decrease in dopamine levels, resulting in neurodevelopmental desordens, accompanied by alterations in Cyp18a1, JAFRAC 1 and TrxR-1 mRNA gene expression, inhibition of the AChE, and -ALA-D activities, as well as changes in Keap-1, and MAPK-2 mRNA gene expressions. Furthermore, it induced modifications in antioxidant enzymes, such as catalase, and glutathione S-transferase activities, along with total thiols content.

Catalase activity (CAT)

As a component of the antioxidant defence system of insects, the enzyme CAT plays a crucial role in catalyzing the conversion of hydrogen peroxide (H_2O_2) into water (H_2O), and molecular oxygen (O_2), this process helps prevent the overproduction of RONS, and H_2O_2, maintaining redox balance under control (Johnson et al. 2021). Furthermore, the CAT also acts in protecting the insect organism against toxic agents, such as botanical larvicides, that can lead to the death of the organism (Shahriari et al. 2018). Indeed, the findings in Fig. 2b indicate clearly that the EO from *T. riparia*, fenchone, and *α*-cypermethrin triggered a significant increase of activity this enzyme in *C. quinquefasciatus* larvae when compared with DMSO ($F\ 3.8 = 285.3$; $p < 0.05$).

These findings corroborate with other studies which reported increase in CAT activity in *D. melanogaster* after exposing to extract at 200 mg/mL from *Cymbopogon citratus* (Poaceae) (Johnson et al. 2021), 4-vinylcyclohexene 1,2-monoepoxide, and 4-vinylcyclohexene diepoxide (Abolaji et al. 2015). Conversely, some insecticides with systemic action, such as neonicotinoid cause the death of insects without inducing alterations in the activity of catalase (Eruslanov and Kusmartsev 2010). For example, the compound imidacloprid did not induce any alterations of CAT in *D. melanogaster*, in comparison with control groups ($p > 0.05$) (Janner et al. 2021).

Glutathione S-transferase activity (GST)

The findings present in Fig. 2c reveal a comparable elevation of GST activity in *C. quinquefasciatus* larvae following exposure to EO from *T. riparia*, and fenchone at a concentration of 25 µg/mL ($p > 0.05$). Additionally, GST activity was highest in *α*-cypermethrin at 0.25 µg/mL than the EO and fenchone ($F\ 3.8 = 97.10$, $p < 0.05$). In contrast, the lower activity was recorded with DMSO assessed at 1%.

The literature data show that changes in the elevation of GST activity have been associated as a significant biomarker of toxicity in vertebrates, and invertebrates exposed to botanical or synthetic larvicides (Domingues et al. 2010; Maestre-Serrano et al. 2014). For example, a significant increase in GST activity, ranging from 15.7 to 100% was identified in *A. aegypti* larvae following exposed to EO, and girgensohnine analog 2-(3,4-dimethoxyphenyl)-2-(piperidin-1-yl)acetonitrile isolates from *Cymbopogon*

flexuosus (Poaceae), while unaltered GST activity was recorded in the negative control ($p < 0.05$) (Carreño Otero et al. 2018).

Certainly, GST constitute another set of phase II detoxification enzymes that protect cell against free radicals, such as RONS (Haddi et al. 2017). They also play a crucial role in catalyzing the conjugation of xenobiotic agents with electrophilic centers with the tripeptide glutathione, resulting a less toxic and more water-soluble product, which facilitates their excretion (Vasantha-Srinivasan et al. 2018).

Acetylcholinesterase (AChE) activity

The exposing of *C. quinquefasciatus* larvae to EO from *T. riparia*, fenchone, and *α*-cypermethrin at a concentration of 25 µg/mL, led to a significant reduction in AChE activity (F 3.8 = 275.3; $p > 0.05$), contrasting with the observed in the control group consisting of DMSO at 1%, that exhibited the highest AChE activity (Fig. 3a). Similar result was recorded upon exposure to a sublethal concentration (LC_{10} of 1.34 ppb) of imidacloprid against *C. quinquefasciatus* larvae for 92 h, that differently modulated the larval biochemical markers, that resulted in decrease of 35.74% of the AChE activity (Koodalingam and Dayanidhi 2021).

The results above confirm that the EO from *T. riparia*, and fenchone act as AChE inhibitors. Indeed, almost that immediately after exposure to EO and fenchone, *C. quinquefasciatus* larvae demonstrated altered movements, and tremors, following death. According to data from literature, AChE is a cholinergic enzyme that catalyses the breakdown of acetylcholine to choline, and acetic acid, thereby terminating nerve transmission at the synapses (Demirci et al. 2017). AChE inhibition result in hyperactivity of excitable tissues, leading to tremors, muscle paralysis, and death (de Lima et al. 2019; Huang et al. 2020). Additionally, research has shown that the toxicity of AChE inhibitors were also correlated with an overproduction of RONS, resulting in oxidative stress (Johnson et al. 2021; de Castro Oliveira et al. 2022).

The altered movements *C. quinquefasciatus* larvae observed in our study were similar to recorded in *Aedes* and *Anopheles* larvae also exposed to EO, methanolic extract from *T. riparia* leaves, and fenchone, in which was reported AChE inhibition of IC_{50} values from 1 mM to 80.8 µg/mL (Amoo et al. 2012; de Oliveira et al. 2022b).

Determination of total thiol content

In terms of thiol content, the ANOVA analysis indicated a significant reduction in the total thiol content in *C. quinquefasciatus* larvae following treatment with EO from *T. riparia*, fenchone, and *α*-cypermethrin, compared to the control group treated with 1% of DMSO (F 3.8 = 184.3; $p < 0.05$), as presented in Fig. 3b.

These results are consistent with those reported by Shahriari et al. (2018), where biochemical assays revealed that monoterpenes *α*-pinene, trans-anethole, and thymol, evaluated at LC_{50} values ranging from 6.81 to 10.66 mg/mL, were able to reduce the thiol content in *Ephestia kuehniella* Zeller (Pyralidae) larvae. In other study, ethanol extract from *C. citratus* leaves, constituted of terpenoids, and evaluated at

concentrations of 100 and 200 mg/mL, caused 70, and 100% mortality of *D. melanogaster*, along with a significant decrease in thiol levels compared to the control group ($p < 0.05$) (Johnson et al. 2021).

Similar to other enzymes in the antioxidant system, thiol constitute a group of enzymes composed of cysteine, homocysteine, glutathione, and cysteinylglycine, distinguished by the presence of a sulfhydryl group, which plays a crucial role in protection by reacting with electrophilic intermediates from RONS (Johnson et al. 2021). Indeed, the reduction in thiol content induced by toxic agents, such as botanical or synthetic larvicides, may expose the larval organism to the toxic effects of these larvicidal products, contributing to mortality (Abolaji et al. 2015).

In this context, we can propose that the reduction in the total thiol content in *C. quinquefasciatus* larvae following treatment with EO from *T. riparia*, fenchone, and *α*-cypermethrin contributed for mortality of this vector larvae.

CONCLUSION

Given the importance of *C. quinquefasciatus* as the primary vector of lymphatic filariasis globally, especially in Brazil. This study highlights that the EO from *T. riparia*, and fenchone, represents a promising larvicides for controlling *C. quinquefasciatus* larvae, posing no lethal effect on the non-target animals studied.

Declarations

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Ethical Approval

The collecting of *T. riparia* was authorized by Sistema de Autorização e Informação em Biodiversidade, No. 78390-1 and registered in the Sistema Nacional de Gestão do Patrimonio Genético e do Conhecimento Tradicional Associado, No. A563032. The collection of non-target animals at the Instituto Nacional de Pesquisas da Amazônia was also authorized by Sistema de Autorização e Informação em Biodiversidade, No. 84157.

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by André C. de Oliveira, Rejane C. Simões, Felipe M. A. da Silva, Ingrity S. C. Sá, Maria L. L. da Costa, Sergio M. Nunomura, Rita C. S. Nunomura and Rosemary A. Roque. The first draft of the manuscript was written by André C. de Oliveira, Felipe M. A. da Silva, and Ingrity S. C. Sá and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose. The author receive

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66. Statements & Declarations

Figures

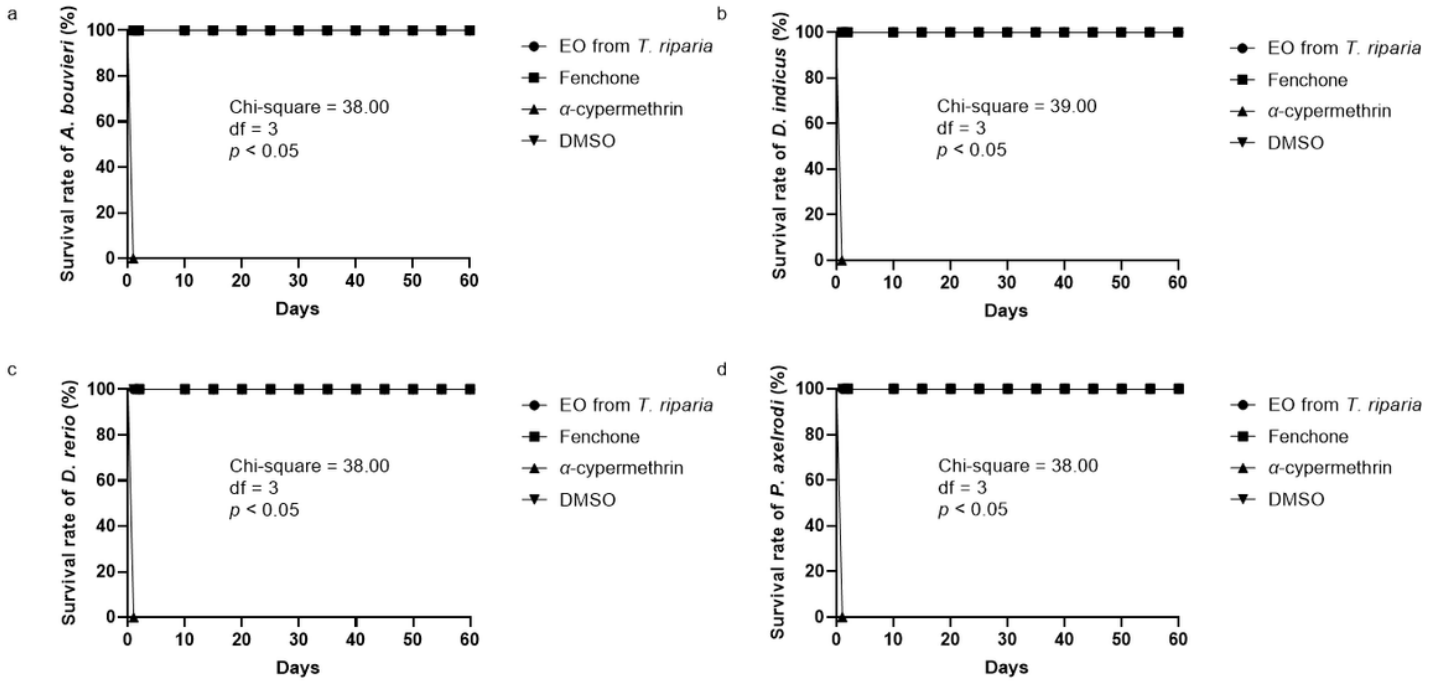


Figure 1

Survival rate of A. bouvieri (a), D. indicus (b), D. rerio (c), and P. axelrodi (d) after 60-day exposure period to concentration of 262.6 and 302.60 µg/mL of the EO from T. riparia, and fenchone, respectively, compared with α-cypermethrin evaluated at 0.25 µg/mL. The data on EO, fenchone, and DMSO overlap. df - Degree of freedom.

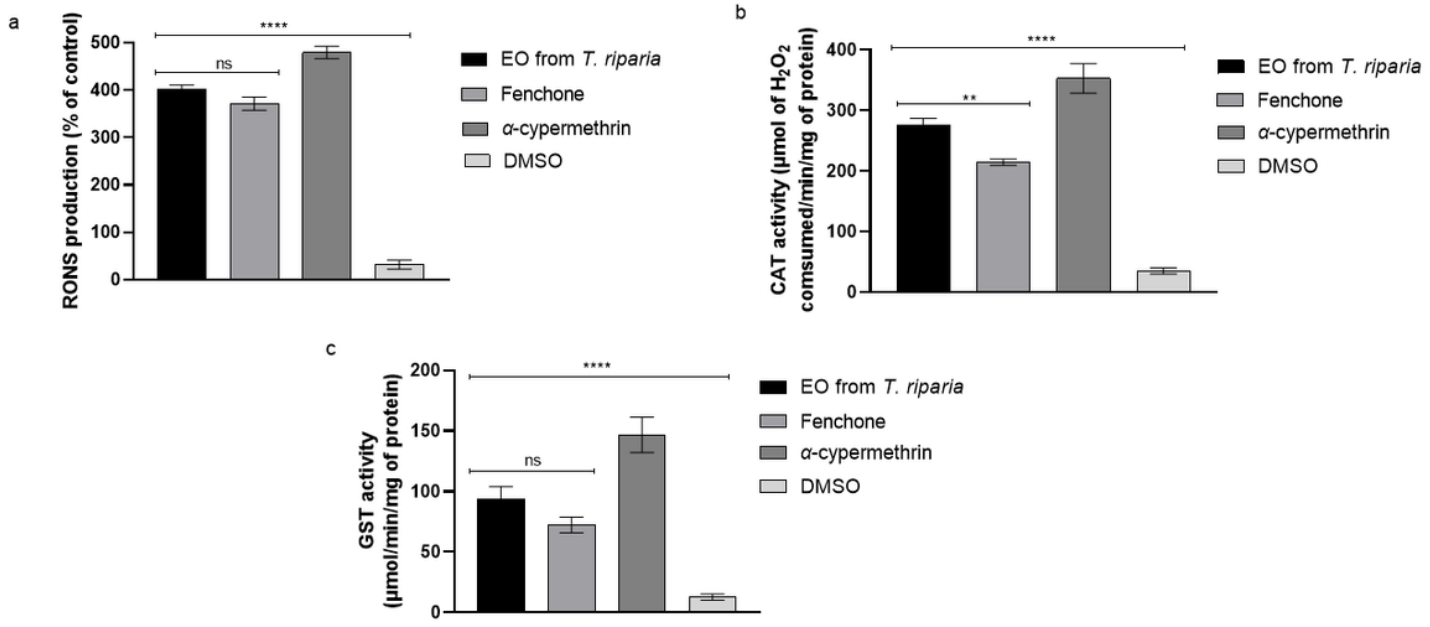


Figure 2

Reactive Oxygen and Nitrogen Species (RONS) production (a) and changes in Catalase (CAT) (b), and Glutathione-S-Transferase (GTS) activity (c) in C. quinquefasciatus larvae after 48-hour period exposing to EO from T. riparia, and fenchone, both assessed at 25 µ/mL, in comparison with the control groups α-cypermethrin at 0.25 µ/mL, and DMSO at 1%. Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$) was employed to identify statistical difference.

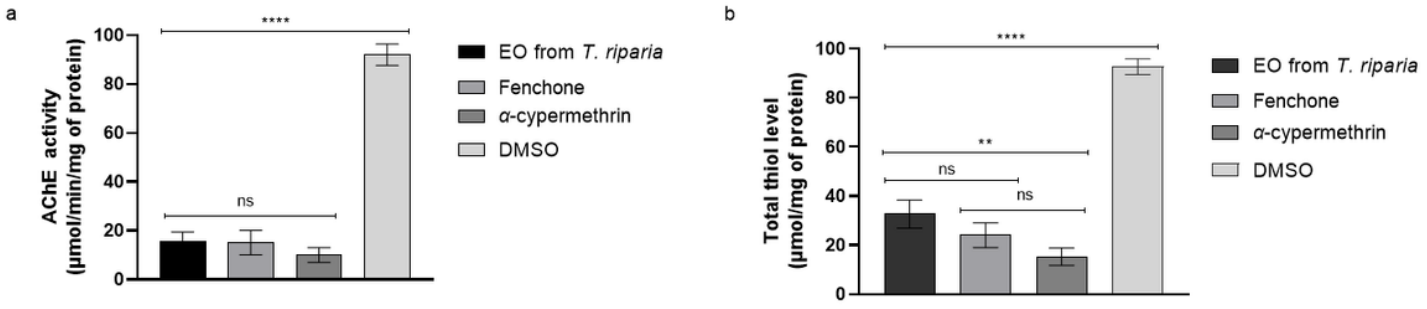


Figure 3

Acetilcolinesterase (AChE) activity (a), and thiol level (b) in C. quinquefasciatuslarvae after exposure to EO from T. riparia, and fenchone, both assessed at (25 µ/mL), in comparison with the control groups α-cypermethrin at 0.25 µ/mL, and DMSO at 1%. Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$) was employed to identify statistical difference.