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POTENTIAL OF *Tetradenia riparia* LEAF ESSENTIAL OIL AND ITS FRACTIONS IN CONTROLLING *Aedes aegypti* AND *Rhipicephalus microplus* LARVAE

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Abstract

Tetradenia riparia (Hochst.) Codd (Lamiaceae) is a shrub, commonly known as ginger bush or false myrrh, and several studies have shown that *T. riparia* exhibits a variety of biological properties. This study aimed to determine the chemical composition of *T. riparia* essential oil and its fractions, investigate their anticholinesterase activity, and assess their larvicidal activity against the cattle tick *Rhipicephalus microplus* and the mosquito *Aedes aegypti*. Eleven essential oil fractions were obtained by fractionation and analyzed by gas chromatography/mass spectrometry. Larvicidal activity against *R. microplus* and third-instar *A. aegypti* was assessed using a larval packet test and a larval immersion test, respectively. Anticholinesterase activity was determined by a bioautographic method. Forty-nine compounds were identified in the essential oil, of which the major classes were oxygenated sesquiterpenes (45.95%) and sesquiterpene hydrocarbons (35.20%) and the major components were isospathulenol (17.40%), β-caryophyllene (15.61%), 14-hydroxy-9-*epi*-caryophyllene (10.07%), 14-hydroxy-α-muurolene (8.32%), and 9β,13β-epoxy-7-abietene (5.53%). Bioassays showed that *T. riparia* essential oil (LC_{50} = 1.56 µg/mL) and FR3 (LC_{50} = 0.30 µg/mL) were the most active against *R. microplus* and *A. aegypti* larvae, respectively. The essential oil and FR1, FR2, and FR3 exhibited acetylcholinesterase inhibitory activity. These results indicate that *T. riparia* essential oil and its fractions hold promise in the development of novel, environmentally safe agents for the control of *R. microplus* and *A. aegypti* larvae.

Keywords: Acetylcholinesterase. Cattle tick. Dengue mosquito. False myrrh.

1. Introduction

Tetradenia riparia (Hochst.) Codd is a shrub belonging to the family Lamiaceae. The plant, commonly known as ginger bush or false myrrh, is native to central Africa and widely used in traditional medicine for the treatment of malaria, angina, headache, tropical skin disease, gastroenteritis, kidney

disease, and fever (Campbell et al. 1997; Martins et al. 2008). In Brazil, the species was introduced as an exotic ornamental plant and became well adapted to the climate, occurring in all Brazilian states in parks, orchards, and residential gardens (Lorenzi and Souza 1999; Martins et al. 2008).

Studies have shown that *T. riparia* exhibits a variety of biological properties, including antispasmodic (Van Puyvelde et al. 1987), antimicrobial (Van Puyvelde et al. 1994), antimalarial (Campbell et al. 1997), antinociceptive (Gazim et al. 2010), antitumor, and antioxidant activities (Gazim et al. 2014). It has also been described as an insecticide (Weaver et al. 1994), repellent against *Anopheles gambiae* (Omolo et al. 2004), acaricide against *Rhipicephalus microplus* (Gazim et al. 2011), and larvicide against *Aedes aegypti* (Fernandez et al. 2014).

For decades, *T. riparia* has been the target of research aiming to identify and isolate compounds found in leaf extracts and essential oil. *Tetradenia riparia* extract was found to contain ibozol, 7α-hydroxyroyleanone (Zelnik et al. 1978), 1',2'-dideacetylboronolide (Van Puyvelde et al. 1981), 8(14),15-sandaracopimaradiene-7α,18-diol (Van Puyvelde et al. 1986), 5,6-dihydro-α-pyrone (umuravumbolide) (Davies-Coleman and Rivett 1995), the tetra dienolide α-pyrone (Van Puyvelde and De Kimpe 1998), abieta-7,9(11)-dien-13-β-ol, 8(14),15-sandaracopimaradiene-2α,18-diol, astragalin, boronolide, and luteolin (Fernandez et al. 2017). 6,7-Dehydroroyleanone (Kasumoto et al. 2009) and 9β,13β-epoxy-7-abietene (Gazim et al. 2014) were isolated from *T. riparia* essential oil.

The use of essential oils to produce ecofriendly insecticides has been widely explored as a strategy to replace conventional synthetic products (Dias and Moraes 2014; Ellse and Wall 2014; Banumathi et al. 2017; Muturi et al. 2019). The intensive and indiscriminate use of synthetic insecticides, including acaricides and larvicides, has generated several environmental problems (contamination of water, soil, animals and their products) and promoted resistance in phytopathogens, pests, and disease vectors (Manjarres-Suarez and Olivero-Verbel 2013; Zara et al. 2016; Banumathi et al. 2017).

Synthetic insecticides, such as organophosphates, play a key role in the control of the cattle tick *Rhipicephalus microplus*, an important ectoparasite of global livestock that causes millions of dollars in losses annually (Grisi et al. 2014), and the mosquito *Aedes aegypti*, an important vector of arboviruses causing a variety of tropical diseases with high mortality and morbidity rates, including dengue, Zika fever, and chikungunya (PAHO/WHO 2020). However, *R. microplus* and *A. aegypti* have developed resistance to the major classes of products currently available, necessitating novel products with different modes of action (Manjarres-Suarez and Olivero-Verbel 2013; Zara et al. 2016; Banumathi et al. 2017). The use of medicinal plants is an interesting alternative in developing countries such as Brazil, underscoring the importance of studies to discover plants with insecticidal effects (Dias and Moraes 2014; Ellse and Wall 2014; Banumathi et al. 2017; Muturi et al. 2019; Bortolucci et al. 2019; Oliveira et al. 2019).

Given the lack of studies on the biological activity of *T. riparia* essential oil fractions and the relevance of identifying natural acaricidal and larvicidal compounds as alternatives to synthetic products, this study aimed to (i) determine the chemical composition of essential oils and fractions of *T. riparia* leaves, (ii) assess their larvicidal potential against *A. aegypti* and *R. microplus*, and (iii) study their mechanism of action through acetylcholinesterase (AChE) inhibition assays.

2. Material and Methods

Plant material

Leaves of *T. riparia* were collected manually in the morning (7:30 to 9:00 a.m.) between March 1, 2014, and April 1, 2015, at the Medicinal Garden of Paranaense University (23°46.225'S 53°16.730'W, 391 m a.s.l.), Umuarama, northwestern Paraná State, Brazil. Botanical identification was carried out by Professor Ezilda Jacomassi, and a voucher specimen was deposited at the Medicinal Garden under number 2502. The species was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under accession number A78B8F0.

Extraction of essential oil

The essential oil was extracted from fresh *T. riparia* leaves (250 g) by hydrodistillation for 3 h using a Clevenger-type apparatus (Gazim et al. 2010). The oil was collected, dried over anhydrous Na_2SO_4, and stored in amber glass flasks at −20 °C until use.

Essential oil fractionation

Tetradenia riparia essential oil was fractionated by glass column chromatography, as described by Gazim et al. (2014). Silica gel 60 (0.063–0.200 mm) was previously activated at 90 °C for 45 min and used as stationary phase at an adsorbent/analyte ratio of 1:25 (w/w). A 5 g aliquot of essential oil was eluted with pentane (100%), pentane/dichloromethane (9:1, 8:2, 7:3, 1:1, and 3:7), dichloromethane (100%), dichloromethane/methanol (9:1, 7:3, and 1:1), and methanol (100%). Each fraction was concentrated under reduced pressure using a rotary evaporator (Tecnal TE-211) at 40 °C. The yield (g) obtained for each fraction is shown in Table 1.

Table 1. Yield of fractions obtained by chromatographic fractionation of *Tetradenia riparia* leaf essential oil.

Fraction	Eluents	Yield (g)
FR1	Pentane (100%)	0.6354
FR2	Pentane/dichloromethane (9:1)	0.6148
FR3	Pentane/dichloromethane (8:2)	0.2414
FR4	Pentane/dichloromethane (7:3)	0.4413
FR5	Pentane/dichloromethane (1:1)	0.4720
FR6	Pentane/dichloromethane (3:7)	0.5410
FR7	Dichloromethane (100%)	0.4744
FR8	Dichloromethane/methanol (9:1)	0.6172
FR9	Dichloromethane/methanol (7:3)	0.2733
FR10	Dichloromethane/methanol (1:1)	0.3547
FR11	Methanol (100%)	0.5876

Gas chromatography/mass spectrometry (GC-MS)

The chemical composition of *T. riparia* essential oil and its fractions was determined using an Agilent 7890B gas chromatograph coupled to an Agilent 5977A MSD mass spectrometer and an Agilent HP5-MS UI fused-silica capillary column (30 m × 250 µm × 0.25 µm; Agilent Technologies). The oven temperature was set at 80 °C for 1 min, increased to 185 °C at 2 °C/min, maintained at this temperature for 1 min, increased to 275 °C at 9 °C/min, maintained for 2 min, and finally increased to 300 °C at 25 °C/min and maintained for 1 min. Helium was used as carrier gas at a constant flow rate of 1 mL/min, 300 °C, and 56 kPa. The injector temperature was set at 280 °C in split mode (2:1 split ratio). The injection volume was 1 µL. The transfer line, ion source, and quadrupole temperatures were 280, 230, and 150 °C, respectively. Detection was performed in scan mode in the *m*/*z* range of 40 to 600 using a solvent delay of 3 min. Compounds were identified by comparison of their mass spectra with the NIST mass spectral database (version 11.0) and by comparison of their retention indices with those of a homologous series of *n*-alkane standards (C7–C28) (Adams 2017).

Larvicidal activity of *T. riparia* essential oil on *R. microplus* larvae

Collection of and rearing of R. microplus individuals

Engorged females of *R. microplus* were collected from dairy cattle at the Veterinary Hospital of Paranaense University, Umuarama, Paraná, Brazil. The cattle had not been treated with acaricides for at least 60 days prior to collection. Ticks were chosen according to their appearance, motility, body integrity, and degree of engorgement (Leite et al. 1995). Individuals were washed in distilled water and weighed; the

mean weight of engorged females was 0.20 g. Subsequently, female ticks were incubated at 27–28 °C and 70–80% relative humidity for 15 days. After this period, the eggs were collected, placed in tubes, and incubated under the same conditions for 15 days. Larvae from tubes with a hatching rate above 95% were used in the experiments.

Larval packet test

The acaricidal activity of *T. riparia* essential oil and its fractions was determined by the Food and Agriculture Organization larval packet test (FAO 2004), performed according to the procedures described by Leite et al. (1995). Essential oil and fractions were diluted to concentrations ranging from 0.005 to 448.28 µg/mL in 2.0% aqueous polysorbate 80 solution. The positive control consisted of a 0.125 µg/mL commercial solution containing 15 µg/mL cypermethrin, 25 µg/mL chlorpyrifos, and 1 µg/mL citronellal. A 2.0% aqueous solution of polysorbate 80 was used as the negative control. *R. microplus* larvae were placed on filter paper envelopes (2.00 × 2.00 cm), which were sealed and impregnated with different concentrations of essential oil and fractions in triplicate. The filter papers were stored in Petri dishes at room temperature for 24 h. After this period, the number of live and dead larvae was counted under an entomological magnifying glass (Leite et al. 1995). Larval mortality (LM) was calculated as follows: LM = [(Dead larvae × 100)/(Total larvae)].

Larvicidal activity on A. aegypti

Bioassays were performed in triplicate using *A. aegypti* larvae obtained from the Control Center for Endemic Vector-Borne Diseases, Health Surveillance Secretariat, Umuarama, Paraná, Brazil. Essential oil and fractions were diluted in 2% aqueous polysorbate 80 to concentrations of 0.005 to 448.28 µg/mL. The larvicidal assay was performed as described by Costa et al. (2005), with modifications. Ten third-instar larvae of *A. aegypti* were collected using a Pasteur pipette and incubated in 250 mL flasks containing 10 mL of the test solutions. Polysorbate 80 (2%) was used as the negative control. The positive control was an organophosphate insecticide (temephos) at 400 µg/mL (Camargo et al. 1998). After 24 h of incubation, the number of live and dead larvae was recorded. Larvae that remained immobile and did not respond to physical stimuli were considered dead. Larval mortality (LM) was calculated as follows: LM = [(Dead larvae × 100)/(Total larvae)].

Anticholinesterase inhibitory activity

Anticholinesterase inhibitory activity was determined by the bioautographic method described by Marston et al. (2002), with modifications (Yang et al. 2009). The essential oil and its fractions were diluted in methanol to concentrations ranging from 0.001 to 448.28 µg/mL. Samples were applied on chromatographic plates (10 × 10 cm, silica gel 60, F254, 0.2 mm thickness), and the plates were left to dry. Plates were then sprayed with a solution of AChE enzyme (500 U) in tris(hydroxymethyl) aminomethane hydrochloride (0.05 M, pH 7.8) and with α-naphthyl acetate (0.15%), incubated at 37 °C for 20 min, and sprayed with Fast Blue B salt reagent (0.05%). Temephos and a 0.125 µg/mL commercial solution containing 15 µg/mL cypermethrin, 25 µg/mL chlorpyrifos, and 1 µg/mL citronellal were used at concentrations ranging from 0.001 to 448.28 µg/mL.

Statistical analysis

All assays were performed in triplicate. Mean and standard deviation values were calculated using Microsoft Excel® version 2010. Data were subjected to analysis of variance (ANOVA) and comparison of means by Tukey's test ($p < 0.05$) using Assistat software version 7.7. Larvicidal activities reported as 50% and 99.9% lethal concentrations (LC_{50} and $LC_{99.9}$), and their respective 95% confidence intervals (CI) were calculated by probit analysis using ED_{50}plus software version 1.0.

3. Results

GC-MS identified 49 compounds in the essential oil of *T. riparia* leaves (Table 2). Sesquiterpenes were the predominant class (81.08%), with isospathulenol (17.40%), β-caryophyllene (15.61%), 14-hydroxy-9-*epi*-caryophyllene (10.07%), 14-hydroxy-α-muurolene (8.32%), and β-cubebene (6.53%) identified as the major compounds.

Chromatographic fractionation afforded 11 fractions (Table 1), 4 of which were active against *A. aegypti* and *R. microplus* larvae. As assessed by GC-MS, fraction 1 (FR1, 7:3 pentane/dichloromethane) was found to contain β-caryophyllene (27.84%), 9β,13β-epoxy-7-abietene (22.11%), 8,13-abietadien-18-ol (12.03%), and 6,7-dehydroroyleanone (7.65%). Fraction 2 (FR2, 3:7 pentane/dichloromethane) was composed of γ-cadinene (68.46%) and germacrene B (26.45%). Fraction 3 (FR3, 100% dichloromethane) contained β-caryophyllene (27.11%), γ-muurolene (22.38%), and α-amorphene (15.66%). Fraction 4 (FR4, 9:1 dichloromethane/methanol) contained limonene (35.02%), δ-cadinene (23.88%), and 14-hydroxy-9-*epi*-caryophyllene (14.24%) (Table 2).

Tetradenia riparia essential oil and its fractions showed larvicidal activity against *A. aegypti* and *R. microplus* in a concentration-dependent manner, as shown in Table 3 and Table 4.

Larval mortality data (Table 3 and Table 4) were used to calculate the LC_{50} and $LC_{99.9}$ by probit analysis (Table 5).

FR3 (LC_{50} = 0.30 µg/mL, $LC_{99.9}$ = 6.48 µg/mL) and FR4 (LC_{50} = 0.61 µg/mL, $LC_{99.9}$ = 1.83 µg/mL) exhibited the highest larvicidal activity against *A. aegypti* larvae, whereas the whole essential oil was the most active against *R. microplus* larvae (LC_{50} = 1.56 µg/mL, $LC_{99.9}$ = 191.53 µg/mL).

The AChE inhibitory effect of *T. riparia* essential oil and its fractions was assessed by visualization of white halos (indicating absence of enzymatic activity) in the bioautographic test. The results of Table 6 show that essential oil, FR1, FR2, and, FR3 had the highest AChE inhibitory activity, inhibiting the enzyme at 0.23, 0.0025, 0.01, and 0.01 µg/mL, respectively. The anti-AChE activities of essential oil, FR2, and FR3 were observed at concentrations similar to their LC_{50} for *A. aegypti* and *R. microplus* larvae (Table 5), suggesting that AChE inhibition may be their mode of action.

4. Discussion

Members of the family Lamiaceae have been extensively investigated, chemically and agronomically, for their richness in essential oils. Research has focused not only on maximizing essential oil extraction but also on identifying and understanding the variation in essential oil composition (Valmorbida et al. 2006).

Gazim et al. (2010) determined the chemical composition of *T. riparia* essential oil obtained from plants occurring in the same region as the specimens used in the present study. The authors investigated the effects of climate on essential oil composition and found that sesquiterpenes constituted the major class of compounds (48.16–69.32%), with 14-hydroxy-9-*epi*-caryophyllene (18.27–24.36%), calyculone (11.57–24.70%), *cis*-muurolol-5-en-4α-ol (7.06–13.78%), and abietadiene (5.51–13.54%) as the major compounds. Similarly, Fernandez et al. (2014) found that sesquiterpenes were the major class (42.42–65.65%) in all seasons and 14-hydroxy-9-*epi*-caryophyllene (11.73–19.62%), calyculone (11.61–25.42%), *cis*-muurolol-5-en-4α-ol (4.46–13.33%), and fenchone (2.93–13.52%) were the major compounds. In the current study, however, oxygenated sesquiterpenes (46.95%) and sesquiterpene hydrocarbons (35.84%) were the predominant classes of compounds in *T. riparia* essential oil; the major compounds were isospathulenol, β-caryophyllene, and 14-hydroxy-9-*epi*-caryophyllene (Table 2).

Our results differed from studies conducted by Gazim et al. (2010) and Fernandez et al. (2014), who did not identify the hydrocarbon sesquiterpenes in significant concentrations.

We investigated which factor could have influenced the chemical composition, since the plant material comes from the same location (Medicinal Garden of Paranaense University (23°46.225'S 53°16.730'W, 391 m a.s.l.), Umuarama, northwestern Paraná State, Brazil). The harvest of plant material also took place in the same period (March and April), coinciding with the end of summer and beginning of autumn, but in different years (2007, 2011 and 2015).

Table 2. Chemical composition of *Tetradenia riparia* leaf essential oil (EO) and its fractions (F1–F4).

Peak	Compound	RI[2]	Relative area (%)					IM
			EO	FR1	FR2	FR3	FR4	
1	α-fenchene	954	t	1.86	-	-	-	a,b,c
2	β-pinene	966	0.35	-	-	-	2.29	a,b,c
3	Myrcene	978	0.50	-	-	-	3.23	a,b,c
4	Limonene	986	4.47	-	-	-	35.02	a,b,c
5	β-phellandrene	996	0.13	-	-	-	1.48	a,b,c
6	δ-3-carene	1110	t			0.97		a,b,c
7	1,8-cineole	1015	0.18	-	0.04	-	6.29	a,b,c
8	γ-terpinene	1048	t	-	0.22	0.94	-	a,b,c
9	Fenchone	1072	1.80	3.62	-	-	1.43	a,b,c
10	*endo*-fenchol	1095	0.23	-	1.11	2.32	-	a,b,c
11	Camphor	1126	0.50	-	1.95	-	-	a,b,c
12	Borneol	1155	1.12	-	-	3.01	1.05	a,b,c
13	α-terpineol	1182	0.86	-	-	-	-	a,b,c
14	Isobornyl acetate	1288	t	1.14	-	-	-	a,b,c
15	α-copaene	1304	0.91	1.61	-	0.45	-	a,b,c
16	Isoledene	1306	0.36	-	-	-	-	a,b,c
17	β-cubebene	1310	6.53	-	-	-	-	a,b,c
18	β-elemene	1320	0.80	-	-	0.35	-	a,b,c
19	α-gurjunene	1322	1.86	-	-	4.43	-	a,b,c
20	β-caryophyllene	1327	15.61	27.84	-	27.11	3.52	a,b,c
21	Aromadendrene	1433	0.30	2.46	-	2.18	-	a,b,c
22	α-humulene	1439	0.83	-	-	-	-	a,b,c
23	Allo-aromadendrene	1448	0.12	-	-	0.80	-	a,b,c
24	γ-gurjunene	1451	0.13	-	-	0.89	-	a,b,c
25	γ-muurolene	1458	0.06	-	-	23.67	-	a,b,c
26	α-amorphene	1459	0.12	-	-	15.66	4.43	a,b,c
27	Germacrene D	1462	1.32	-	-	-	-	a,b,c
28	β-selinene	1464	0.52	1.89	-	0.67	-	a,b,c
29	*cis*-β-guaiene	1472	0.06	-	-	-	-	a,b,c
30	Valencene	1477	0.63	-	-	-	-	a,b,c
31	α-muurolene	1479	1.39	-	-	-	-	a,b,c
32	γ-cadinene	1481	0.27	6.06	68.46	-	1.98	a,b,c
33	δ-cadinene	1487	0.23	5.67	-	0.56	23.88	a,b,c
34	Germacrene B	1501	3.08	-	26.45	-	1.15	a,b,c
35	Spathulenol	1508	0.27	-	-	-	-	a,b,c
36	Globulol	1510	0.57	-	-	-	-	a,b,c
37	Viridiflorol	1514	0.19	-	-	-	-	a,b,c
38	Isospathulenol	1519	17.40	-	0.19	0.67	-	a,b
39	α-muurolol	1627	2.81	-	-	-	-	a,b,c
40	α-cadinol	1646	5.82	-	-	-	-	a,b,c
41	14-hydroxy-9-*epi*-caryophyllene	1667	10.07	-	-	-	14.24	a,b,c
42	*cis*-farnesol	1670	0.50	-	-	-	-	a,b,c
43	14-hydroxy-α-muurolene	1782	8.32	-	-	-	-	a,b,c
44	Abietatriene	2031	0.22	-	-	-	-	a,b,c
45	9β,13β-epoxy-7-abietene	2071	5.53	22.11	0.71	1.10	-	d
46	8,13-abietadien-18-ol	2304	0.46	12.03	0.18	2.04	-	a,b,c
47	Abietol	2420	0.20	-	-	-	-	a,b,c
48	Manoyl oxide	2461	0.25	2.74	-	0.45	-	a,b,c
49	6,7-dehydroroyleanone	2482	0.33	7.65	0.09	6.29	-	d
	Total		98.21	96.68	99.40	94.56	99.99	
	Monoterpene hydrocarbons		5.45	1.86	0.22	1.91	42.02	
	Oxygenated monoterpenes		4.69	4.76	3.10	5.33	8.77	
	Sesquiterpene hydrocarbons		35.13	45.53	94.91	76.77	34.96	
	Oxygenated sesquiterpenes		45.95	-	0.19	0.67	14.24	
	Diterpene hydrocarbons		0.22	-	-	-	-	
	Oxygenated diterpenes		6.77	44.53	0.98	9.88	-	

[a]RI = identification based on the calculated retention index (RI) utilizing a standard homologous series of *n*-alkanes C_7-C_{26} in HP-5MS UI column; [b]Compounds = compounds listed in elution order in HP-5MS UI column; [c]identification based on the comparison of mass spectra found in NIST 11.0 libraries; [d]identification by nuclear magnetic resonance imaging (Gazim et al. 2014); Relative area (%) = percentage of the area occupied by compounds in the chromatogram; t= traces; (-) = not detected. IM = identification methods.

Table 3. Mortality rate (%) of *Aedes aegypti* larvae treated with *Tetradenia riparia* leaf essential oil and its fractions.

Concentration (µg/mL)	Mortality rate (%)				
	EO	FR1	FR2	FR3	FR4
PC	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
448.28	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
244.14	100.00 ± 0.00^{a}	81.66 ± 0.70^{b}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
122.07	100.00 ± 0.00^{a}	60.00 ± 0.00^{c}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
61.03	100.00 ± 0.00^{a}	35.41 ± 0.78^{d}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
30.51	100.00 ± 0.00^{a}	0.00 ± 0.00^{e}	77.50 ± 0.98^{b}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
15.25	100.00 ± 0.00^{a}	0.00 ± 0.00^{e}	63.33 ± 1.41^{c}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
7.62	60.00 ± 0.00^{b}	0.00 ± 0.00^{e}	53.27 ± 1.52^{d}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
3.81	52.27 ± 0.77^{c}	0.00 ± 0.00^{e}	35.41 ± 0.78^{e}	77.50 ± 0.98^{b}	100.00 ± 0.00^{a}
1.90	17.14 ± 1.16^{d}	0.00 ± 0.00^{e}	24.28 ± 0.50^{f}	70.71 ± 0.24^{c}	100.00 ± 0.00^{a}
0.95	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	64.58 ± 0.78^{d}	60.00 ± 0.00^{b}
0.47	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	60.00 ± 0.00^{e}	50.00 ± 0.00^{c}
0.23	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	52.57 ± 0.82^{f}	41.42 ± 0.58^{d}
0.11	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	35.41 ± 1.57^{g}	33.33 ± 0.00^{e}
0.05	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	26.78 ± 0.65^{h}	20.00 ± 0.00^{f}
0.02	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	20.00 ± 0.00^{i}	0.00 ± 0.00^{g}
0.01	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	13.39 ± 0.27^{j}	0.00 ± 0.00^{g}
0.005	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	0.00 ± 0.00^{k}	0.00 ± 0.00^{g}
NC	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	0.00 ± 0.00^{k}	0.00 ± 0.00^{g}

EO, essential oil. FR1, fraction 1 (7:3 pentane/dichloromethane), which contains β-caryophyllene (37.76%), 8,13-abietadien-18-ol (29.99%), manoyl oxide (16.32%), germacrene D (8.22%), and germacrene B (7.69%). FR2, fraction 2 (3:7 pentane/dichloromethane), which contains α-muurolol (42.39%), germacrene B (28.58%), α-cadinol (11.03%), δ-cadinene (9.52%), and isoledene (8.48%). FR3, fraction 3 (dichloromethane), which contains 14-hydroxy-9-*epi*-caryophyllene (38.04%), γ-muurolene (31.40%), δ-gurjunene (21.97%), and germacrene D (8.57%). FR4, fraction 4 (9:1 dichloromethane/methanol), which contains limonene (44.09%), δ-cadinene (30.06%), 14-hydroxy-9-*epi*-caryophyllene (17.93%), and 1,8-cineole (7.91%). PC, positive control (commercial solution based on an organophosphorus compound). NC, negative control (20 mL/L v/v aqueous polysorbate 80 solution). Values are the mean ± standard deviation of an experiment carried out in triplicate. Data were subjected to analysis of variance (ANOVA), and differences between means were assessed by Tukey's test ($p \leq 0.05$). Different letters in the same column indicate significant differences between treatments ($p \leq 0.05$).

Table 4. Mortality rate (%) of *Rhipicephalus microplus* larvae treated with *Tetradenia riparia* leaf essential oil and its fractions.

Concentration (µg/mL)	Mortality rate (%)				
	EO	FR1	FR2	FR3	FR4
PC	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
976.56	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
448.28	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	84.65 ± 0.77^{b}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
244.14	100.00 ± 0.00^{a}	70.50 ± 0.12^{b}	80.93 ± 0.75^{c}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
122.07	91.66 ± 0.46^{b}	50.00 ± 0.00^{c}	75.71 ± 0.56^{d}	60.85 ± 0.97^{b}	59.41 ± 0.13^{b}
61.03	89.19 ± 0.14^{b,c}	39.50 ± 0.14^{d}	50.86 ± 0.17^{e}	55.70 ± 0.11^{c}	58.57 ± 0.06^{b}
30.51	86.66 ± 0.12^{b,c}	11.50 ± 0.14^{e}	36.93 ± 0.18^{f}	34.18 ± 0.44^{d}	36.60 ± 0.05^{c}
15.25	84.99 ± 0.30^{c}	0.00 ± 0.00^{f}	30.94 ± 0.95^{g}	29.85 ± 0.17^{e}	12.37 ± 0.28^{d}
7.62	75.12 ± 0.47^{d}	0.00 ± 0.00^{f}	25.00 ± 0.00^{h}	25.17 ± 0.60^{f}	7.17 ± 0.13^{e}
3.81	67.50 ± 2.08^{e}	0.00 ± 0.00^{f}	23.21 ± 0.43^{i}	20.00 ± 0.00^{g}	7.10 ± 0.26^{e}
1.90	64.44 ± 5.83e	0.00 ± 0.00^{f}	14.16 ± 0.19^{j}	13.95 ± 0.06^{h}	0.00 ± 0.00^{f}
0.95	48.01 ± 2.58^{f}	0.00 ± 0.00^{f}	11.66 ± 0.33^{k}	6.45 ± 0.05^{i}	0.00 ± 0.00^{f}
0.47	13.39 ± 0.22^{g}	0.00 ± 0.00^{f}	0.00 ± 0.00^{l}	0.00 ± 0.00^{j}	0.00 ± 0.00^{f}
NC	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}	0.00 ± 0.00^{l}	0.00 ± 0.00^{j}	0.00 ± 0.00^{f}

EO, essential oil. FR1, fraction 1 (7:3 pentane/dichloromethane), which contains β-caryophyllene (37.76%), 8,13-abietadien-18-ol (29.99%), manoyl oxide (16.32%), germacrene D (8.22%), and germacrene B (7.69%). FR2, fraction 2 (3:7 pentane/dichloromethane), which contains α-muurolol (42.39%), germacrene B (28.58%), α-cadinol (11.03%), δ-cadinene (9.52%), and isoledene (8.48%). FR3, fraction 3 (dichloromethane), which contains 14-hydroxy-9-*epi*-caryophyllene (38.04%), γ-muurolene (31.40%), δ-gurjunene (21.97%), and germacrene D (8.57%). FR4, fraction 4 (9:1 dichloromethane/methanol), which contains limonene (44.09%), δ-cadinene (30.06%), 14-hydroxy-9-*epi*-caryophyllene (17.93%), and 1,8-cineole (7.91%). PC, positive control (0.125 µg/mL commercial solution containing 15 µg/mL cypermethrin, 25 µg/mL

chlorpyrifos, and 1 µg/mL citronellal in 2% polysorbate 80); NC, negative control (20 mL/L v/v aqueous polysorbate 80 solution). Values are the mean ± standard deviation of an experiment carried out in triplicate. Data were subjected to analysis of variance (ANOVA), and differences between means were assessed by Tukey's test ($p \leq 0.05$). Different letters in the same column indicate significant differences between treatments ($p \leq 0.05$).

Table 5. Lethal concentrations (LC_{50} and $LC_{99.9}$) and confidence limits of *Tetradenia riparia* leaf essential oil and its fractions against *Rhipicephalus microplus* and *Aedes aegypti* larvae, as calculated by probit analysis.

Sample	*Aedes aegypti*		*Rhipicephalus microplus*	
	LC_{50} (LCL–UCL)	$LC_{99.9}$ (LCL–UCL)	LC_{50} (LCL–UCL)	$LC_{99.9}$ (LCL–UCL)
EO	5.60[c] (5.55–5.64)	14.41[c] (14.33–14.47)	1.56[d] (1.25–1.86)	191.53[d] (183.34–199.71)
FR1	100.57[a] (99.23–101.91)	395.75[a] (391.59–399.90)	149.80[a] (148.77–150.82)	413.33[b] (411.70–414.66)
FR2	9.83[b] (9.11–10.54)	53.54[b] (52.18–54.69)	65.56[c] (59.66–71.45)	768.55[a] (746.82–790.27)
FR3	0.30[e] (0.29–0.31)	6.48[d] (6.34–6.62)	78.16[b] (73.08–83.23)	229.60[c] (224.77–234.44)
FR4	0.61[d] (0.60–0.62)	1.83[e] (1.82–1.84)	81.19[b] (80.95–81.42)	226.22[c] (225.09–227.35)

EO, essential oil. FR1, fraction 1 (7:3 pentane/dichloromethane), which contains β-caryophyllene (37.76%), 8,13-abietadien-18-ol (29.99%), manoyl oxide (16.32%), germacrene D (8.22%), and germacrene B (7.69%). FR2, fraction 2 (3:7 pentane/dichloromethane), which contains α-muurolol (42.39%), germacrene B (28.58%), α-cadinol (11.03%), δ-cadinene (9.52%), and isoledene (8.48%). FR3, fraction 3 (dichloromethane), which contains 14-hydroxy-9-*epi*-caryophyllene (38.04%), γ-muurolene (31.40%), δ-gurjunene (21.97%), and germacrene D (8.57%). FR4, fraction 4 (9:1 dichloromethane/methanol), which contains limonene (44.09%), δ-cadinene (30.06%), 14-hydroxy-9-*epi*-caryophyllene (17.93%), and 1,8-cineole (7.91%). LC_{50}, lethal concentration that kills 50% of the exposed larvae. $LC_{99.9}$, lethal concentration that kills 99% of the exposed larvae. LCL, lower confidence limit. UCL, upper confidence limit. Values are the mean ± standard deviation of an experiment carried out in triplicate. Data were subjected to analysis of variance (ANOVA), and differences between means were assessed by Tukey's test ($p \leq 0.05$). Different letters in the same column indicate significant differences between treatments ($p \leq 0.05$).

Table 6. Acetylcholinesterase inhibitory activity of *Tetradenia riparia* leaf essential oil and its fractions, as assessed by a bioautographic method.

Concentration (µg/mL)	Inhibitory activity						
	EO	FR1	FR2	FR3	FR4	PC1	PC2
448.28	+	+	+	+	+	+	+
244.14	+	+	+	+	-	+	+
122.07	+	+	+	+	-	+	+
61.03	+	+	+	+	-	+	+
30.51	+	+	+	+	-	+	+
15.25	+	+	+	+	-	+	+
7.62	+	+	+	+	-	+	+
3.81	+	+	+	+	-	+	+
1.90	+	+	+	+	-	+	+
0.95	+	+	+	+	-	+	+
0.47	+	+	+	+	-	-	-
0.23	+	+	+	+	-	-	-
0.11	-	+	+	+	-	-	-
0.05	-	+	+	+	-	-	-
0.02	-	+	+	+	-	-	-
0.01	-	+	+	+	-	-	-
0.005	-	+	-	-	-	-	-
0.0025	-	+	-	-	-	-	-
0.001	-	-	-	-	-	-	-

EO, essential oil. FR1, fraction 1 (7:3 pentane/dichloromethane), which contains β-caryophyllene (37.76%), 8,13-abietadien-18-ol (29.99%), manoyl oxide (16.32%), germacrene D (8.22%), and germacrene B (7.69%). FR2, fraction 2 (3:7 pentane/dichloromethane), which contains α-muurolol (42.39%), germacrene B (28.58%), α-cadinol (11.03%), δ-cadinene (9.52%), and isoledene (8.48%). FR3, fraction 3 (dichloromethane), which contains 14-hydroxy-9-*epi*-caryophyllene (38.04%), γ-muurolene (31.40%), δ-gurjunene (21.97%), and germacrene D (8.57%). FR4, fraction 4 (9:1 dichloromethane/methanol), which contains limonene (44.09%), δ-cadinene (30.06%), 14-hydroxy-9-*epi*-

caryophyllene (17.93%), and 1,8-cineole (7.91%). PC1, positive control 1 (organothiophosphate temephos). PC2, positive control 2 (0.125 µg/mL commercial solution containing 15 µg/mL cypermethrin, 25 µg/mL chlorpyrifos, and 1 µg/mL citronellal in 2% polysorbate 80).

Analyzing the climatic conditions, we found that there was a similarity between Gazim et al. (2010) and Fernandez et al. (2014) with temperatures of (28 to 30ºC; 21 to 26ºC); rainfall index of (150 to 200mm and 136 to 159 mm), respectively.

When comparing the climatic data with our experiment with temperatures ranging from (18.73 to 28.8ºC) and rainfall index (66.98 to 72.04 mm), it was possible to observe lower rainfall in the period of March and April 2015, when this experiment was conducted. This low rainfall may be responsible for the change in chemical composition, causing an increase in sesquiterpenes hydrocarbons (35.13%) and oxygenated (45.95%), especially the compound β-caryophyllene (27.84%) (Table 2). This hypothesis is supported by studies conducted by Palmer-Youn et al. (2015), where the production of sesquiterpenes is increased when the plant is subjected to water stress, which occurred in our experiment due to the low rainfall. These authors also state that (E)-β-caryophyllene is exceptionally reactive with atmospheric oxidants, particularly ozone.

Such differences in essential oil composition reflected on biological activity. The larvicidal activity of the essential oil obtained in the present study against *A. aegypti* (LC_{50} = 5.60 µg/mL) and *R. microplus* (LC_{50} = 1.56 µg/mL) (Table 5) was superior to that of the essential oil obtained by Fernandez et al. (2014). The authors evaluated the larvicidal activity of *T. riparia* essential oil against *A. aegypti* as a function of season and observed that the activity was highest in fall (LC_{50} = 78.72 µg/mL) and spring (LC_{50} = 83.29 µg/mL). Gazim et al. (2011) reported that the LC_{50} of *T. riparia* essential oil for *R. microplus* larvae was 1220.00 µg/mL. According to Morais (2009), environmental stimuli can alter metabolic pathways in plants, thereby modifying the biosynthesis of compounds and biological activity. It is likely that the difference in biological activity between essential oils is due to differences in chemical composition.

Isospathulenol and 14-hydroxy-9-*epi*-caryophyllene were two of the major compounds in *T. riparia* essential oil; their larvicidal effects on *A. aegypti* and *R. microplus*, however, have not been previously reported. β-Caryophyllene exhibited larvicidal activity against *A. aegypti* (LC_{50} = 88.30 µg/mL), *Culex pipiens pallens* (LC_{50} = 93.65 µg/mL), and *Ochlerotatus togoi* (LC_{50} = 97.90 µg/mL) (Perumalsamy et al. 2009). There are no reports of the acaricidal activity of β-caryophyllene on *R. microplus*, but the compound was shown to be effective against the mites *Dermatophagoides farinae* (LC_{50} = 3.13 µg/cm^2), *Dermatophagoides pteronyssinus* (LC_{50} = 3.58 µg/cm^2) (Oh et al. 2014), *Tyrophagus putrescentiae* (LC_{50} = 11.77 µg/cm^2) (Kim et al. 2003), and *Tetranychus urticae* (LC_{50} = 0.00080 µg/mL) (Cavalcanti et al. 2010), indicating that β-caryophyllene contributed to the larvicidal and acaricidal action of the essential oil.

Compounds with an LC_{50} lower than 100 µg/mL are considered active against *A. aegypti* larvae and those with an LC_{50} lower than 50 µg/mL are classified as highly active (Cheng et al. 2003). According to this classification, *T. riparia* essential oil and FR2, FR3, and FR4 were highly active against *A. aegypti*, whereas FR1 was active.

The dichloromethane fraction (FR3) exhibited the highest *A. aegypti* larvicidal activity (LC_{50} = 0.30 µg/mL) (Table 5). Its anti-AChE activity was correlated with its larvicidal action, suggesting that the larvicidal mode of action is related to AChE inhibition, affecting cholinergic neurotransmission. This fraction contained sesquiterpene hydrocarbons, particularly germacrene D, which is known to act against *A. aegypti* (LC_{50} = 18.76 µg/mL), *Anopheles stephensi* (LC_{50} = 16.95 µg/mL), and *Culex quinquefasciatus* (LC_{50} = 21.28 µg/mL) larvae (Govindarajan 2010). Germacrene D was also found to be toxic by fumigation to the malaria mosquito *A. gambiae* (LC_{50} 1.8 × 10^{-3} mg/cm^3), *C. quinquefasciatus* (LC_{50} = 2.1 × 10^{-3} mg/cm^3), and *A. aegypti* (LD_{50} = 2.8 × 10^{-3} mg/cm^3) (Kiran and Devi 2007).

FR4 (9:1 dichloromethane/methanol) was also highly active (LC_{50} = 0.61 µg/mL) against *A. aegypti* larvae (Table 5). The fraction was composed mainly of limonene, a precursor of monoterpene biosynthesis. In a study by Cheng et al. (2008), limonene was shown to be the most effective against *A. aegypti* (LC_{50} = 19.41 µg/mL) and *Aedes albopictus* (LC_{50} = 15.00 µg/mL). Botas et al. (2017) developed a D-limonene nanoemulsion with larvicidal potential against *A. aegypti* (LC_{50} = 81.19 µg/mL). FR4 also contained δ-cadinene (Table 2), a compound previously shown to exert larvicidal effects on *A. aegypti* (LC_{50} = 9.03 µg/mL), *A. stephensi* (LC_{50} = 8.23 µg/mL), and *C. quinquefasciatus* (LC_{50} = 9.86 µg/mL) (Govindarajan et al. 2016).

FR4 showed low anti-AChE activity (448.28 µg/mL) (Table 6), and there was no relationship between biological activity and AChE inhibitory activity. Such findings may suggest that the mechanism of action of FR4 is like that of pyrethroid insecticides, which act by preventing the closure of sodium channels and disrupting the balance of sodium and potassium ions in axonal membranes, thereby impairing nerve impulse transmission in *R. microplus* and *A. aegypti* larvae (Ware and Whitacre 2000).

FR3 and FR4 were more active against *A. aegypti* larvae than their isolated compounds (reported in previous studies). Essential oil fractions are a mixture of terpenes, which may interact synergistically to increase larvicidal activity (Araújo et al. 2016; Dhinakaran et al. 2019), as was evidenced in our study. Thus, a combination of terpenes is more effective than isolated compounds.

In the anti-AChE assay, FR1 differed from the other fractions in that it showed high activity (0.0025 µg/mL) (Table 6); however, its larvicidal activity against *R. microplus* (LC_{50} = 149.80 µg/mL) and *A. aegypti* (LC_{50} = 100.57 µg/mL) (Table 5) was low. High molecular weight oxygenated sesquiterpenes and diterpenes were identified in FR1, which may explain its low larvicidal effects. According to Benson (2005) and Brain et al. (2007), low molecular weight molecules with adequate hydrophilic and lipophilic solubility are easily absorbed by larval cells, but a high molecular weight may hinder penetration.

Tetradenia riparia essential oil and its fractions exhibited important larvicidal activity on *R. microplus* and *A. aegypti*, showing promise as an alternative to chemical larvicides. Furthermore, *T. riparia* essential oil has low cytotoxicity (Cardoso et al. 2015), an important factor for its application in the control of *R. microplus* and *A. aegypti*.

5. Conclusions

The major components of *T. riparia* essential oil were isospathulenol, β-caryophyllene, and 14-hydroxy-9-*epi*-caryophyllene. The essential oil and some of its fractions showed larvicidal activity against *R. microplus* and *A. aegypti* as well as anticholinesterase activity. FR3 was the most active against *A. aegypti* larvae, whereas the whole essential oil had the highest larvicidal potential against *R. microplus*. These findings suggest that *T. riparia* essential oil is an interesting candidate for the development of environmentally friendly agents to control *R. microplus* and *A. aegypti* larvae.

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