Revista Brasileira de Parasitologia Veterinária
Brazilian Journal of Veterinary Parasitology
ISSN 1984-2961 (Electronic)
www.cbpv.org.br/rbpv

Original Article

Activity of essential oils from leaves, flower buds and stems of *Tetradenia riparia* on *Rhipicephalus* (*Boophilus*) *microplus* larvae

Atividade dos óleos essenciais das folhas, botões florais e caules de *Tetradenia riparia* sobre as larvas de *Rhipicephalus (Boophilus) microplus*

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How to cite: Cella W, Rahal IL, Silva GCC, Jacomassi E, Piau Junior R, Gonçalves JE, et al. Activity of essential oils from leaves, flower buds and stems of *Tetradenia riparia* on *Rhipicephalus* (*Boophilus*) *microplus* larvae. *Braz J Vet Parasitol* 2023; 32(1): e013522. https://doi.org/10.1590/S1984-29612023011

Abstract

Around the world, the main problems of livestock are caused by ectoparasites, however, commercial acaracide are toxic to the environment and detrimental to One Health. Therefore, research has increasingly focused on development of natural products as alternatives for tick control. The purpose of this study was to evaluate the larvicidal effect on *Rhipicephalus (Boophilus) microplus*, through use of essential oils (EOs) extracted from the leaves, flower buds and stems of *Tetradenia riparia*. The chemical composition of these EOs was determined through gas chromatography coupled to mass spectrometry (GC-MS). They were tested on larvae at concentrations of 100.000 to 40 µg/mL, using the larval packet test and under semi-natural conditions. The main class of compounds in the chemical composition was sesquiterpenes (both oxygenates and hydrocarbons), whereas the predominant compounds in the leaves, flower buds and stems were 14-hydroxy-9-epi-caryophyllene, T-cadinol and 6-7-dehydroroyleanone, respectively. The leaves proved to be the most effective, with highest larvicidal activity ($LC_{99.9}$ = 83.53 µg/mL). When tested under semi-natural conditions, the oils obtained efficiency above 98% in all compound tests. The results indicated that these EOs were effective against *R. (B.) microplus* larvae *in vitro* and *ex-situ*, proving that this plant has bioactive molecules with significant larvicidal activity.

Keywords: Larvicide, essential oils, ticks, *Tetradenia riparia*, *Rhipicephalus microplus*.

Resumo

Os principais problemas para a pecuária estão relacionados às ectoparasitoses, e ao fato dos carrapaticidas apresentarem elevada toxicidade ao meio ambiente e à saúde única. Surgem, então, demandas na busca por inovações e desenvolvimento de produtos naturais, como alternativas para o controle dos carrapatos. O objetivo deste trabalho foi avaliar o potencial da atividade larvicida sobre *Rhipicephalus (Boophilus) microplus* a partir dos óleos essenciais de *Tetradenia riparia* (TrOEs) extraídos das folhas, botões florais e caules. A composição química foi determinada por cromatografia gasosa acoplada à espectrometria de massa (GC/MS). Os TrOEs foram testados sobre larvas nas concentrações de 100.000 a 40 µg/mL pelo teste de pacote de larvas e em condições seminaturais. Na composição química, a classe majoritária foi os sesquiterpenos (oxigenados e hidrocarbonetos);

Received October 7, 2022. Accepted January 25, 2023.
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já os compostos em destaques foram 14-hidroxy-9-epi-caryophyllene, T-cadinol e 6-7-dehidroroyleanone para folhas, botões florais e caules, respectivamente. As folhas demonstraram ser mais eficientes e com maior poder larvicida ($CL_{99.9}$ = 83.53 µg/mL). Quando testado em condições seminaturais os óleos obtiveram eficiência acima de 98% em todos os compostos testados. Os resultados indicaram que os TrOEs, foram eficazes sobre as larvas de *R. (B.) microplus in vitro* e *ex-situ,* evidenciando que esta planta possui moléculas bioativas com ação larvicidas significativas.

Palavras-chave: Larvicida, óleo essencial, carrapatos, *Tetradenia riparia*, *Rhipicephalus microplus*.

Introduction

Brazil has the largest commercial herd of cattle in the world, with approximately 214.8 million head. It is currently the fourth largest producer of milk, producing about 25.4 billion liters/year. The livestock sector accounts for 8.5% of Brazil's gross domestic product (GDP) (IBGE, 2020), which shows the strength of this sector in the Brazilian economy. However, farmers suffer productivity losses relating to ectoparasitosis, especially due to infestations of *Rhipicephalus* (*Boophilus*) *microplus* Canestrini, 1887 (Acari: Ixodidae). This ectoparasite is responsible for causing losses in Brazil of the order of US$ 3.2 billion/year (Grisi et al., 2014). Worldwide, ticks are among the main pests of livestock. Experts estimate that losses due to tick infestations could reach US$ 30 billion/year (Lew-Tabor & Valle, 2016).

Most of the negative economic impacts arise through direct expenditure on treating the damage that ticks cause to the animals' health, such as blood loss, dermatitis, immune suppression, food inappetence and stress. These are due mainly to a complex of diseases known as cattle tick fever, which are transmitted by these ticks (Araújo et al., 2015; Garcia et al., 2019). The consequences of cattle tick fever include weight loss, reduced milk production, falling birth rates, leather depreciation and increased mortality (Andreotti et al., 2019). Moreover, indirect expenditure is incurred with regard to the cost of labor, acquisition of acaricide products and investments in infrastructure, equipment and logistics that are used exclusively in combating ticks.

Although there are a multitude of methods for controlling these ectoparasites, farmers always use methods that are easy to apply, with lower costs. These characteristics are commonly found among manufactured chemicals (Higa et al., 2019), especially pyrethroids and organophosphates (Mendes et al., 2013).

Given the magnitude of the losses, the fact that commercial acaricides present high toxicity to the environment, animals and humans and the high standards demanded within international markets, there is a need for research relating to innovation and development of natural products. The aim is to search for economically viable alternatives that promote strategic tick control, combined with sustainable development throughout the production chain (Chagas et al., 2002; Medeiros et al., 2019).

Among the various species with potential for the development of bioactive products, *Tetradenia riparia* stands out. This is a shrub plant belonging to the family Lamiaceae that is native to the African continent but which has been introduced and duly adapted to all Brazilian biomes. It is used as an ornamental and aromatic plant, and is popularly known as false myrrh, lavandula or mist plum (Gazim et al., 2011). Essential oils (EOs) with different types of biological activity such as antimalarial, antibacterial, fungicidal, anthelmintic and acaricide activity can be extracted from this plant (Campbell et al., 1997; Gazim et al., 2011; Kakande et al., 2019; Van Puyvelde et al., 2018).

The aim of the present study was to evaluate the potential for larvicidal activity on *R.* (*B.*) *microplus* larvae, through use of *T. riparia* essential oils (EOs) extracted from the following botanical structures: leaves, flower buds and stems.

Materials and Methods

Plant material

Botanical structures (leaves, flower buds and stems) of *T. riparia* were collected in the winter season (May-July) of 2020. The plants had been cultivated in the Medicinal Garden of Universidade Paranaense (UNIPAR), located in the city of Umuarama, northwestern region of the state of Parana, Brazil (latitude 23° 46′ 22″ S, longitude 53° 16′ 73″ W; altitude 391 m). The plant was identified by Professor Ezilda Jacomasi of the Department of Pharmacy of Paranaense Univeresity (UNIPAR), Paraná. A voucher specimen was deposited at the UNIPAR Herbarium (code number 2502). This species is registered in the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the registration number AFFE469.

Obtaining essential oils

Tetradenia riparia essential oils (EOs) were obtained separately from different botanical structures (leaves, flower buds and stems). All the plant material was dried at room temperature in a covered and ventilated place and was then ground up in a knife mill. The extraction process consisted of hydrodistillation for three hours in a modified Clevenger apparatus (Gazim et al., 2010). After extraction and separation, the essential oils (EOs) were stored in amber flasks and kept under refrigeration at 4 °C until the time of the experiment. The essential oil yield was determined through calculating the ratio of the mass of dry leaves, fresh flower buds and stems (g) divided by the mass of essential oil (g) (%).

Chemical identification of essential oils

Chemical identification of the EOs was performed by means of gas chromatography-mass spectrometry (GC-MS) using a gas chromatograph (Agilent 7890B) coupled to a mass spectrometer, (Agilent 5977A MSD) equipped with capillary column HP5-MS UI 5% (30 × 250 µm × 0.25 µm; Agilent Technologies) and with automatic injector (CTC PAL Control). In order to perform a proper separation of the analytes in the GC-MS system, 2 µL of EO were injected into the column using Split injection mode in a 1:30 ratio, with injector temperature of 260 °C, flow of carrier gas He of 1 mL min-1 and under the following oven conditions: initial temperature of 80 °C, then ramp from 4 °C min-1 to 260 °C and lastly ramp from 40 °C min-1 to 300 °C. The transfer line was kept at 280 °C and the ionization source and quadrupole at 230 °C and 150 °C, respectively. The detection system was EM (Gain 1.5), in scan mode, in the mass-to-charge ratio range of 40 to 550 m/z, with a "solvent delay" of 3 min. The volatile compounds were identified by comparisons with mass spectra found with the mass spectra from the NIST Library version 11.0 and with retention indices (RI) that the been obtained from a homologous series of n-alkane (C7-C40) standards (Adams, 2017).

Physicochemical indexes of essential oils from *T*. *riparia*.

The refractive index was determined in an ABBE refractometer, model RL3, brand PZO Warszawa at 20 °C (Korolkovas et al., 1988).

The specific optical rotation was determined using an ACA TEC automated polarimeter (Korolkovas et al., 1988). The solvent used for dilution of the EOs was absolute alcohol. The concentration of the EOs from leaves and stems used in this test was 0.156% and the concentration from flower buds was 0.039%, at a temperature of 20 °C, using a 10 cm tube.

The absolute density (g/mL) was determined in a 5 µL graduated capillary tube and was calculated as the ratio of the mass (g) of EOs divided by the volume (mL) that this oil occupied in the capillary tube, both at 20 °C, in accordance with the technique previously described (Korolkovas et al., 1988).

Principal Component Analysis (PCA)

Multivariate exploratory evaluation in the form of principal component analysis (PCA) was conducted. This enabled joint evaluation of the major chemical compounds and the chemical classes of all compounds present in the essential oils of the leaves, flower buds and stems. The results from the analysis were presented in graphic form (Biplot), which helped to characterize the groups of variables analyzed (Moita & Moita, 1998). For each sample of EOs obtained from the leaves, flower buds and stems, the major chemical compounds identified, their respective chemical classes and the amounts (area % on the plots) were transformed into orthogonal latent variables called principal components, which were linear combinations of the original variables created, with the eigenvalues of the data covariance matrix. The Kaiser criterion was used to select the principal components. Their eigenvalues preserved relevant information when they were greater than unity (Camacho et al., 2010; Ferré, 1995). This analysis was performed in two ways: the first contained only the data referring to the major compounds identified in the EOs of the leaves, flower buds and stems. The second contained the data referring to the chemical classes to which these compounds belonged. Both analyses were performed using the Statistica 13 software (StatSoft Inc.).

Collection of engorged females

Engorged females of *R*. (*B*) *microplus* were randomly collected (n ≅ 300) from naturally infested dairy cattle belonging to a farm in the municipality of São Tomé, northwestern region of the state of Parana, Brazil (latitude

23° 31' 22" S; longitude 52° 35' 08" W). On this farm, the cattle were only receiving homeopathic treatments and, at the time of tick collection, they had gone approximately 240 days without receiving any treatment.

The ticks collected were transported in a container with adequate aeration to the UNIPAR Parasitology Laboratory. In the laboratory, only the engorged females were selected, on the basis of normal appearance and motility, intact body and maximum engorgement with homogeneous weight (Medeiros et al., 2019). These were placed, without treatment, in a chamber with temperature-controlled environmental conditions that resembled the tick's natural environment: approximately 28 °C; relative humidity (RH) ≥ 80%; and light/dark photoperiods of 12 h each. The aim was to produce larvae for the experimental tests. The average age of larvae used in the experiments was 21 days after hatching.

In vitro larvicidal activity of essential oils from *T. riparia*

For the *R.* (*B.*) *microplus* larvae to undergo the larval packet test (LPT), they were placed in sealed filter paper envelopes (Stone & Haydock, 1962), as adapted by Monteiro et al. (2012). The EOs were applied homogeneously at concentrations of (100,000; 50,000; 25,000; 12,500; 6,250; 3,120; 1,560; 780; 390; 190; 95 and 40 µg/mL) (mass/volume) using 2.00% polysorbate 80 (v/v) as the emulsifier and purified water as the solvent (Chagas et al., 2012). An aqueous solution of 2.00% polysorbate 80 (v/v) was used as a negative control (NC). A commercial solution at 0.125% (v/v) containing 15.00% cypermethrin, 25.00% chlorpyrifos and 1.00% citronellal was used as a positive control (PC). Larvae that were found to be immobile upon being touched, 24 hours after application of the solution, were considered dead. The percentage larval mortality was calculated from this (Equation 1).

$$Mortality\ (\%) = (dead\ larvae\ x\ 100) / total\ larvae \quad (1)$$

The lethal concentrations (LCs) that killed 99.9% ($LC_{99.9}$) of the tick larvae population, with their respective confidence intervals (CI), were calculated by means of Probit analysis (ED 50 Plus, version 1.0). All tests were performed in triplicate. The EO concentrations that gave rise to $LC_{99.9}$, obtained from the LPT, were used in a subsequent test for *ex situ* control of ectoparasites in plastic pots.

Larvicidal activity of essential oils from *T. riparia* under semi-natural conditions

Plastic pots (n = 15) of dimensions 25 cm in height and 25 cm in diameter were each filled with 2.20 kg of soil and seedlings of the grass species *Brachiaria decumbens* were planted. The plants were kept in a greenhouse for three months with irrigation. At the time of the tests, the leaves of *B. decumbens* were cut 40 cm from the soil surface, and adhesive tape was placed around the rim of each pots as a physical barrier to contain the larvae of *R.* (*B.*) *microplus* (40 mg) which were deposited on the soil surface of each plant pot. After 24 h, it was observed that the larvae had migrated to the apex of the grass leaves (Araújo et al., 2015). Each group of three pots constituted one treatment. The $LC_{99.9}$ obtained through the LPT for the EOs from the leaves, flower buds and stems were used for the treated group, plus 20.00% mineral oil, 2.00% polysorbate 80 (v/v) and distilled water qsp (sufficient quantity), up to a volume of 15.00 mL.

A commercial acaricide solution was used as the positive control and an aqueous solution of 2.00% polysorbate 80 (mass/volume) plus 20.0% mineral oil was used as the negative control. For each treatment, 5.00 mL of each solution was sprayed on separately, in the respective pots, starting at the top of the plant and going down to the base only once. This simulated application of commercial acaricides to pastures. After 24 h, the leaves of *B. decumbens* were cut and, with the aid of a stereomicroscope, the larvae were counted. Larvae that did not show motility upon being touched were considered dead. Next, the mean numbers of live larvae in the NC group and in the group of larvae treated with EOs were determined. From these data, the percentage efficiency of the EOs was calculated, as shown in Equation 2, as described by Bittencourt et al. (2003).

$$Efficiency\ of\ TrEOs\ (\%) = \left[(A - B) / A\right] x\ 100 \quad (2)$$

Where A = average number of live larvae in the negative control group and B = average number of live larvae in the groups treated with TrEOs.

Statistical analysis

The experimental design was completely randomized (DIC). Data were subjected to analysis of variance (ANOVA) and differences between arithmetic means and standard deviation were determined using Tukey's test, with a significance level of 5%. The lethal concentrations (LCs) that killed 50.0% ($LC_{50.0}$) and 99.9% ($LC_{99.9}$) of *R.* (*B.*) *microplus* larvae and their respective CIs were calculated by means of Probit analysis (ED 50 Plus version 1.0). All tests were performed in triplicate.

Results

The essential oils extracted from *T. riparia* (EOs) have a characteristic smell of incense, and their color ranges in intensity from lighter orange (stems) to darker orange in the leaves and reddish orange in the flower buds. The absolute density (g/mL), specific optical rotation, refractive index and yield (%) are shown in Table 1. The chemical composition of the EOs obtained through GC-MS indicated the presence of 61 compounds in the leaves, 49 in the flower buds and 55 in the stems. Compounds in the class of sesquiterpenes (both oxygenates and hydrocarbons) were most abundant, accounting for 61.12%, 60.68% and 67.01% of the compounds found in leaves, flower buds and stems, respectively (Table 2).

Table 1. Physicochemical indices for absolute density (g/mL), refractive index, specific optical rotation and yield (%), of the essential oils from the leaves, flower buds and stems of *Tetradenia riparia*.

Botanical Structures (EOs)	Physicochemical indices			
	Refractive index n_D^{20}CI	Specific optical rotation $\lvert\alpha\rvert_D^{20}$	Absolute density (g/mL) d_{20}^{20}CI	Yield (%) CI
Leaves	1.4971 ± 0.0008^{C}	- 12.01°	0.83 ± 0.05^{A}	0.86 ± 0.01^{A}
	(1.4947 - 1.4996)		(0.78 - 0.87)	(0.84 - 0.89)
Flower buds	1.5056 ± 0.0028^{B}	- 26.33°	0.90 ± 0.00^{A}	0.33 ± 0.03^{B}
	(1.5032 - 1.5081)		(0.85 - 0.95)	(0.31- 0.36)
Stems	1.5588 ± 0.0025^{A}	- 24.48°	0.88 ± 0.05^{A}	0.13 ± 0.02^{C}
	(1.5563 - 1.5612)		(0.83 - 0.92)	(0.11 - 0.15)

EOs: essential oils; CI: confidence interval. Means followed by same letters in the same column do not statistically differ according to Tukey's test at the significance level of $p < 0.05$.

Table 2. Chemical composition of the essential oils from the leaves, flower buds and stems of *Tetradenia riparia*.

Peak	RT	Compounds	MF	MW	RI Lit	RI calc	Leaves	Floral buds	Stems	Identification methods
		Hydrocarbon monoterpenes					Relative area (%)			
1	3.180	α-pinene	C10H16	136	932	902	1.01	-	2.03	a, b, c
2	3.194	Camphene	C10H16	136	946	955	0.92	1.74	2.04	a, b, c
3	3.316	β-pinene	C10H16	136	964	961	-	0.36	1.25	a, b, c
4	3.456	Sabinene	C10H16	136	969	967	-	-	0.08	a, b, c
5	3.530	α-phellandrene	C10H16	136	996	1008	0.37	-	-	a, b, c
6	3.648	α-terpinene	C10H16	136	1014	1014	0.04	-	-	a, b, c

a: compounds listed according to their elution order in an HP-5 MS column; b: retention index (RI) calculated using n-alkanes C9 to C30 in an HP-5 MS column; c: identification based on comparison with mass spectrum from NIST 11.0 libraries; relative area (%): percentage of the area occupied by the compounds in the chromatogram; n.i.: not identified; RT: retention time; (-): compounds absent; MF: molecular formula; MW: molecular weight.

Table 2. Continued...

Peak	RT	Compounds	MF	MW	RI Lit	RI calc	Leaves	Floral buds	Stems	Identification methods
		Hydrocarbon monoterpenes					Relative area (%)			
7	3.752	D-limonene	C10H16	136	1024	1019	2.09	1.15	0.33	a, b, c
8	4.196	β-phellandrene	C10H16	136	1026	1020	0.04	1.66	-	a, b, c
9	4.239	trans-β-ocimene	C10H16	136	1050	1044	0.06	1.53	-	a, b, c
10	4.310	n.i				1047	-	0.74	-	a, b, c
11	4.737	γ-terpinene	C10H16	136	1064	1065	0.11	0.21	-	a, b, c
							4.64	**6.65**	**5.73**	
		Oxygenated monoterpenes								
12	4.901	cis-sabinene hydrate	C10H18O	154	1068	1071	0.47	0.21	-	a, b, c
13	5.352	Fenchone	C10H16O	152	1088	1088	7.09	4.33	4.51	a, b, c
14	5.832	Fenchol, exo	C10H18O	154	1112	1114	1.17	1.2	0.09	a, b, c
15	6.474	n.i				1137	0.31	-	-	a, b, c
16	6.530	Camphor	C10H16O	152	1143	1139	2.18	2.85	0.11	a, b, c
17	7.009	endo-borneol	C10H18O	154	1166	1159	0.86	1.42	1.76	a, b, c
18	7.278	α-terpineol	C10H18O	154	1189	1170	0.32	0.39	-	a, b, c
19	7.594	Terpinen-4-ol	C10H18O	154	1206	1215	0.69	0.85	0.1	a, b, c
							12.78	**11.25**	**6.57**	
		Other compounds								
20	8.895	Borneol formate	C11H18O2	182	1232	1234	-	-	0.14	a, b, c
21	10.458	Bornyl acetate	C12H20O2	196	1285	1266	-	-	0.27	a, b, c
									0.41	
		Hydrocarbon sesquiterpenes								
22	11.533	Elixene	C15H24	204		1328	0.51	0.39	0.53	a, b, c
23	12.296	n.i				1355	0.05	-	0.11	a, b, c
34	12.657	α-copaene	C15H24	204	1374	1367	0.53	1.56	0.36	a, b, c
25	13.114	β-elemene	C15H24	204	1394	1382	0.91	0.85	-	a, b, c
26	13.365	α-gurjunene	C15H24	204	1408	1390	1.28	2.38	1.18	a, b, c
27	13.944	Caryophyllene	C15H24	204	1418	1409	5.84	6.37	6.35	a, b, c
28	14.356	β-farnesene	C15H24	204	1429	1424	0.77	-	0.37	a, b, c
29	14.582	β-copaene	C15H24	204	1430	1433	-	-	0.38	a, b, c
30	14.707	α-bergamotene	C15H24	204	1435	1437	0.6	0.91	0.52	a, b, c
31	14.888	Aromadendrene	C15H24	204	1440	1443	-	-	0.13	a, b, c

a: compounds listed according to their elution order in an HP-5 MS column; b: retention index (RI) calculated using n-alkanes C9 to C30 in an HP-5 MS column; c: identification based on comparison with mass spectrum from NIST 11.0 libraries; relative area (%): percentage of the area occupied by the compounds in the chromatogram; n.i.: not identified; RT: retention time; (-): compounds absent; MF: molecular formula; MW: molecular weight.

Table 2. Continued...

Peak	RT	Compounds	MF	MW	RI Lit	RI calc	Leaves	Floral buds	Stems	Identification methods
		Hydrocarbon monoterpenes					Relative area (%)			
32	14.922	β-gurjunene	C15H24	204	1442	1444	-	-	2.77	a, b, c
33	15.027	Humulene	C15H24	204	1455	1448	0.52	0.44	0.17	a, b, c
34	15.027	Alloaromadendrene	C15H24	204	1455	1448	0.18	0.51	0.39	a, b, c
35	15.237	n.i				1455	0.09	-	-	a, b, c
36	15.334	γ-elemene	C15H24	204	1465	1461	0.4	0.8	1.47	a, b, c
37	15.545	γ-muurolene	C15H24	204	1477	1466	4	1.8	4.05	a, b, c
38	15.560	Eremophilene	C15H24	204	1486	1477	0.53	0.82	2.81	a, b, c
39	16.068	β-guaiene	C15H24	204	1488	1483	-	-	1.33	a, b, c
49	16.143	α-muurolene	C15H24	204	1499	1485	1.55	4.52	0.64	a, b, c
41	16.230	α-bulnesene	C15H24	204	1508	1494	0.21	0.6	0.74	a, b, c
42	16.405	α-farnesene	C15H24	204	1509	1501	-	1.17	0.32	a, b, c
43	16.605	β-cadinene	C15H24	204	1520	1507	0.55	1.74	3.37	a, b, c
44	16.800	δ-cadinene	C15H24	204	1524	1511	-	4.9	-	a, b, c
							22.38	**29.76**	**27.88**	
		Oxygenated Sesquiterpenes								
45	16.905	6-epi-shyobunol	C15H26O	222	1505	1488	3.51	1.71	1.69	a, b, c
46	17.614	Elemol	C15H26O	222	1549	1537	0.41	-	-	a, b, c
47	17.896	Palustrol	C15H26O	222	1567	1547	0.51	0.3	-	a, b, c
48	18.054	n.i				1553	0.13	-	1.27	a, b, c
49	18.324	Spathulenol	C15H24O	220	1571	1562	1.9	0.28	0.64	a, b, c
50	18.432	Caryophyllene oxide	C15H24O	220	1578	1566	4.71	3.11	3.43	a, b, c
51	18.818	Globulol	C15H26O	222	1584	1579	0.13	0.93	5.83	a, b, c
52	19.386	Viridiflorol	C15H26O	222	1593	1598	-	0.83	1.01	a, b, c
53	19.446	n.i				1600	-	-	0.31	a, b, c
54	19.598	Isoaromadendrene epoxide	C15H24O	220	1594	1606	0.43	1.57	2.2	a, b, c
55	19.921	α-acorenol	C15H26O	222	1632	1618	1.25	2.92	1.46	a, b, c
56	20.173	n.i				1628	0.25	-	0.31	a, b, c
57	20.290	T-cadinol	C15H26O	222	1640	1632	3.6	9.66	5.22	a, b, c
58	20.325	n.i				1633	-	0.23	-	a, b, c
59	20.547	Cubenol	C15H26O	222	1643	1641	0.62	-	2.55	a, b, c
60	21.047	α-Eudesmol	C15H26O	222	1652	1660	3.95	-	3.99	a, b, c

a: compounds listed according to their elution order in an HP-5 MS column; b: retention index (RI) calculated using n-alkanes C9 to C30 in an HP-5 MS column; c: identification based on comparison with mass spectrum from NIST 11.0 libraries; relative area (%): percentage of the area occupied by the compounds in the chromatogram; n.i.: not identified; RT: retention time; (-): compounds absent; MF: molecular formula; MW: molecular weight.

Table 2. Continued...

Peak	RT	Compounds	MF	MW	RI Lit	RI calc	Leaves	Floral buds	Stems	Identification methods
		Hydrocarbon monoterpenes					Relative area (%)			
61	21.239	α-Cadinol	C15H26O	222	1653	1666	7.56		4.85	a, b, c
62	22.056	14-hydroxy-9-epi-caryophyllene	C15H24O	220	1678	1695	10.16	9.61	6.26	a, b, c
							38.74	**30.92**	**39.13**	
		Diterpenes (Hydrocarbon and Oxygenated)								
63	22.381	Abieta-8,11,13-triene	C20H28O		1682	1701	0.17	-	1.04	a, b, c
64	27.593	Abietadiene	C20H32	220	1714	1711	0.34	-	0.1	a, b, c
65	28.361	Cembrene	C20H32	272	1939	1943	0.14	0.51	0.18	a, b, c
66	29.046	Isophytol	C20H40O	296	1942	1972	0.56	-	-	a, b, c
67	29.406	L labda-8(20),12,14-triene	C20H32	272	1962	1986	0.34	-	-	a, b, c
68	29.461	9β,13β-epoxy-7-abietene	C20H32O	288		1988	6.47	6.86	5.4	a, b, c
69	29.556	Sclarene	C20H32	272	1964	1992	0.39	0.39	-	a, b, c
70	29.821	M manoyl oxide	C20H34O	290	1998	2003	0.45	0.26	0.26	a, b, c
71	30.537	Abieta-8(14),9(11),12-triene	C20H30	270	2073	2035	2.09	3.54	0.68	a, b, c
72	33.339	Sclareol	C20H36O2	308	2229	2259	0.88	0.53	0.11	a, b, c
73	33.434	6-7-dehydroroyleanone	C20H32O	288	2253	2263	7.27	7.64	9.58	a, b, c
74	36.836	n.i	C20H30O	286	2314	2323	0.09	-	-	a, b, c
75	37.177	n.i		314	2466	2439	0.27	0.38	-	a, b, c
							20.1	**19.73**	**17.35**	
		Hydrocarbon monoterpenes					4.64	6.65	5.73	
		Oxygenated monoterpenes					12.78	11.25	6.57	
		Hydrocarbon sesquiterpenes					22.38	29.76	27.88	
		Oxygenated sesquiterpenes					38.74	30.92	39.13	
		Hydrocarbon diterpenes					3.30	4.44	0.96	
		Oxygenated diterpenes					15.80	15.29	16.39	
		Other compounds					0	0	0.41	
		Unidentified					1.19	1.35	2	

a: compounds listed according to their elution order in an HP-5 MS column; b: retention index (RI) calculated using n-alkanes C9 to C30 in an HP-5 MS column; c: identification based on comparison with mass spectrum from NIST 11.0 libraries; relative area (%): percentage of the area occupied by the compounds in the chromatogram; n.i.: not identified; RT: retention time; (-): compounds absent; MF: molecular formula; MW: molecular weight.

Class projections from principal component analysis (PCA) indicated that factor 1 accounted for 97.02% of the variability of the EOs found in the different botanical structures. EOs from leaves and stems exhibited a vector with a smaller angle, thus revealing that these structures were positively correlated with the class of oxygenated sesquiterpenes (38.74% and 39.13%, respectively). Moreover, the EOs of the flower buds and stems showed a positive correlation with the class of hydrocarbon sesquiterpenes (29.76% and 27.88%, respectively) (Figure 1 and Table 2).

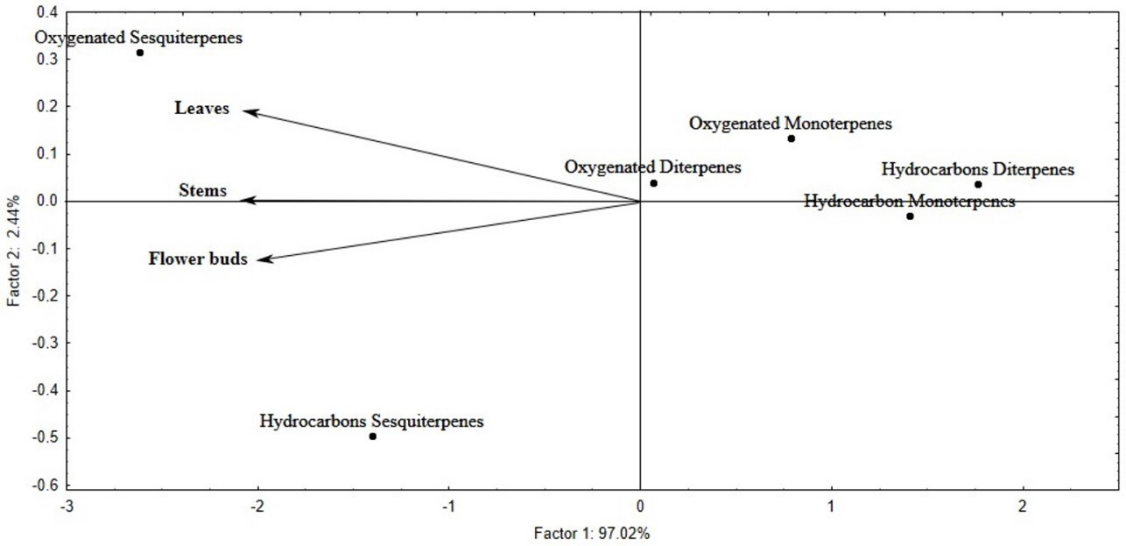

Figure 1. Biplot representing the projection of chemical classes of essential oils from the leaves, flower buds and stems of *Tetradenia riparia*, obtained by means of gas chromatography coupled to mass spectrometry (GC/MS).

The projections from PCA on compound groupings indicated that factor 1 accounted for 67.28% of the variability of the TrEOs found in the different botanical structures. The EOs of the leaves and stems did not show angle separation and indicated that they were absolutely correlated with α-cadinol compounds (7.56% and 4.85%, respectively), fenchone (7.09% and 4.51%) and 6-7-dehydroroyleanone (7.27% and 9.58%). Although the EOs of the flower buds exhibited a vector with a large angle in relation to the other structures, there were positive correlations with the compounds 14-hydroxy-9-epi-caryophyllene (10.16%, 9.61% and 6.26%), caryophyllene (5.84%, 6.37% and 6.35%) and 9β,13β-epoxy-7-abietene (6.47%, 6.86% and 5.40%) for the leaves, flower buds and stems, respectively (Figure 2 and Table 2).

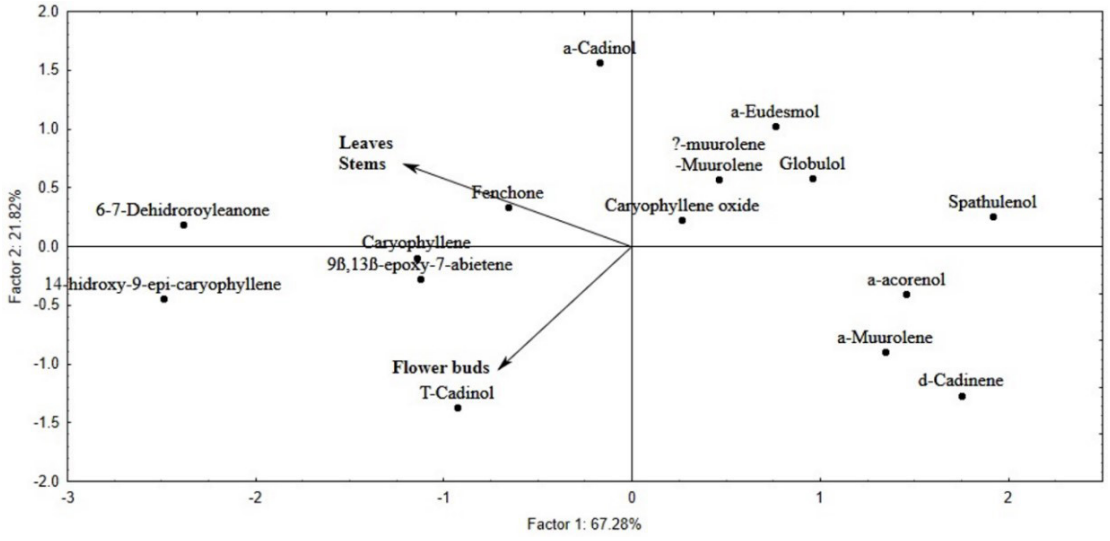

Figure 2. Biplot representing the projection of major compounds in the essential oils from the leaves, flower buds and stems of *Tetradenia riparia*, obtained by means of gas chromatography coupled to mass spectrometry (GC/MS).

The results regarding the larvicidal potential of *R*. (*B*.) *microplus* exposed to different concentrations of EOs through the LPT test indicated that the EOs from the botanical structures of the leaves, flower buds and stems yielded high mortality rates at concentrations of 100,000 and 50,000 µg/mL, without significant differences in relation to the PC, which killed 100% of the larvae.

At the concentration of 25,000 µg/mL, the leaves and stems presented mortality rates that were considered high (80.54% and 91.63%, respectively), without any significant difference between these structures. However, the floral buds showed worse performance (66.36%). It is important to point out that the negative control showed zero percentage mortality with regard to EOs from all three botanical structures, and that this result differed significantly from the other results (Table 3).

Table 3. Mortality rate (%) among larvae of *Rhipicephalus (Boophilus) microplus* subjected to treatments with essential oils from the leaves, flower buds and stems of *Tetradenia riparia*, at different concentrations.

Concentrations (µg/mL)	Botanical structures (EOs)		
	Leaves	Flower buds	Stems
PC	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
100,000	100.00 ± 0.00^{aA}	95.90 ± 3.91^{aA}	98.64 ± 2.17^{aA}
50,000	97.21 ± 4.27^{aA}	92.01 ± 8.57^{aA}	95.63 ± 4.58^{aA}
25,000	80.54 ± 4.26^{bA}	66.36 ± 9.14^{bB}	91.63 ± 10.30^{aA}
12,500	62.76 ± 9.14^{cB}	51.89 ± 3.40^{cC}	78.07 ± 2.10^{bA}
6,250	47.02 ± 5.80^{dB}	42.49 ± 8.11^{cdB}	67.67 ± 6.05^{cA}
3,120	41.38 ± 3.62^{dA}	37.95 ± 3.92^{dA}	37.71 ± 4.80^{dA}
1,560	29.38 ± 5.60^{eAB}	35.87 ± 8.02^{dA}	21.60 ± 3.67^{eB}
780	18.82 ± 4.77^{fB}	33.91 ± 7.98^{dA}	15.32 ± 1.68^{efB}
390	15.04 ± 2.03^{fgB}	19.26 ± 2.50^{eA}	14.71 ± 1.13^{efB}
190	12.02 ± 2.44^{fgA}	16.29 ± 4.59^{eA}	13.85 ± 3.26^{efA}
95	6.13 ± 0.69^{ghC}	13.13 ± 1.94^{efA}	9.30 ± 1.25^{fgB}
40	1.09 ± 0.72^{hA}	1.43 ± 3.19^{fgA}	1.04 ± 0.69^{ghA}
NC	0.00 ± 0.00^{hA}	0.00 ± 0.00^{gA}	0.00 ± 0.00^{hA}

EOs: essential oils; PC: positive control; NC: negative control. The lowercase letters between lines. Capital letters between columns. Means followed by same letters do not statistically differ according to Tukey's test at the significance level of $p < 0.05$.

The lethal concentrations (LC) were determined by means of Probit analysis. The results showed that the TrEOs from the leaves were most effective, requiring the lowest concentration ($LC_{99.9}$ = 83.53 µg/mL), in comparison with the other botanical structures: floral buds and stems showed ($LC_{99.9}$ = 91.91 and 85.42 µg/mL), respectively. In all treatments, $LC_{99.9}$ were significantly different for positive control (Table 4).

After all $LC_{99.9}$ values had been determined (Table 4), further assays were performed under semi-natural conditions (*ex situ*) to determine the product efficiency (PE) of the TrEOs used in each treatment. The effectiveness of the TrEOs from all the botanical structures (leaves, flower buds and stems) were found to be 98.25%, 98.48% and 98.14%, respectively. These values were not significantly different from the positive control, which showed efficacy of 100%. The negative control showed zero percent PE, which was significantly different from all the treatments (Table 5).

Discussion

The EOs presented yields of between 0.13% and 0.86%, and the OE of the leaves was the one that presented the highest yield (0.86%) (Table 1). Comparatively, in similar studies on EOs extracted from *T. riparia* leaves, yields of

Table 4. Lethal concentrations ($LC_{50.0}$ and $LC_{99.9}$) in µg/mL, as the arithmetic mean with standard deviation and confidence interval (CI), of the essential oils from leaves, flower buds and stems of *Tetradenia riparia* on larvae of *Rhipicephalus (Boophilus) microplus* by Probit analysis.

Botanical structures (EOs)	$LC_{50.0}$ (CI)	$LC_{99.9}$ (CI)
PC	2.068 ± 0.0010^{A}	19.055 ± 0.0015^{A}
	(2.0665 - 2.0694)	(19.052 - 19.059)
Leaves	7.43 ± 1.07^{B}	83.53 ± 3.07^{B}
	(5.88 - 8.77)	(80.45 - 88.19)
Flower buds	11.55 ± 2.72^{C}	91.91 ± 8.13^{B}
	(7.36 - 14.42)	(81.26 - 101.94)
Stems	5.58 ± 0.60^{AB}	85.42 ± 9.74^{B}
	(4.77 - 6.06)	(74.11 - 98.78)

EOs: essential oils; $LC_{50.0}$: lethal concentration 50.0%; $LC_{99.9}$: lethal concentration 99.9%; CI: confidence interval (α = 0.05). Means followed by different letters in the same column differ statistically between treatments according to Tukey's test at the significance level of $p < 0.05$.

Table 5. Mean lethal concentration ($LC_{99.9}$ µg/mL), average number of live larvae (ALL) in pot tests (ex situ) and product efficiency (PE%) of the essential oils from the leaves, flower buds and stems of *Tetradenia riparia*, acting against the larvae of *Rhipicephalus (Boophilus) microplus*, determined through Probit analysis.

Botanical structures (EOs)	Product efficiency		
	$LC_{99.9}$ (CI)	ALL (CI)	PE (%)
PC	19.055	0.00 ± 0.00	100.00^{A}
	(19.052 - 19.059)	(0.00 - 0.00)	
Leaves	83.53 ± 3.07	5.00 ± 0.00	98.25^{A}
	(80.45 - 88.19)	(1.64 - 8.35)	
Flower buds	91.91 ± 8.13	5.66 ± 0.58	98.48^{A}
	(81.26 - 101.94)	(2.31 - 9.02)	
Stems	85.42 ± 9.74	5.33 ± 0.58	98.14^{A}
	(74.11 - 98.78)	(1.97 - 8.68)	
NC	-	286.66 ± 5.77	00.00^{B}
		(283.31 - 290.02)	

Means followed by same letters in the same column do not statistically differ between treatments according to Tukey's test at the significance level of $p < 0.05$. EOs: essential oils; $LC_{99.9}$: lethal concentration 99.9%; CI: confidence interval; PE: product efficiency; PC: positive control, commercial organophosphorus (0.125% v/v) containing 150.00 mg/mL of cypermethrin, 250.00 mg/mL of chlorpyrifos and 10.00 mg/mL of citronellal; NC: negative control, aqueous solution of polysorbate 80 (2% v/v).

between 0.26% and 0.29% were found (Cardoso et al., 2015; Gazim et al., 2010; Zardeto-Sabec et al., 2020). These previous studies all used leaf structures collected in winter, which was also the seasonal period of the present study.

Araújo (2014) evaluated the effect of luminosity on *T. riparia* and found that the maximum yield of EOs was 0.26% in situations of 30% shading, thus proving the effect of light incidence on the yield of EOs. The availability of solar radiation interferes with the leaf morphophysiology of plants and is strongly related to abiotic factors (Araújo et al., 2019). Consequently, it influences the yield of EOs from many plant species. This would explain the different rates of TrEO yields found by different authors.

The physicochemical parameters of the EOs from all botanical structures were also analyzed, with the following results: absolute density (AD) between 0.83 and 0.90 (g/mL); refractive index (RI) between 1.4971 and 1.5588; and specific optical rotation (SOR) between -12.01° and -26.33°. The ranges of these parameters were equivalent to those

found in the literature for EOs extracted from *T. riparia* leaves, regarding AD 0.88 to 0.95 (g/mL) and RI (1.2916 to 1.4685), but the range of SOR found here was significantly different to what had previously been reported in the literature (Campbell et al., 1997; Gazim et al., 2010). Hypothetically, these variations in SOR can be explained in terms of differences between geographical regions, seasonal periods and climatic and edaphic conditions, in relation to those of the present study (Table 1). Nevertheless, these indices need to be taken into account in industrial-scale production, in order to determine the final quality of the product, in view of the instability of EOs in relation to light, humidity, heat and oxygen levels (Medeiros et al., 2019).

The EOs from the leaves, flower buds and stems showed a complex mixture of chemical compounds. The analyses performed by means of GC/MS showed that the largest class of compounds identified were sesquiterpenes (both hydrocarbons and oxygenates), which accounted for 61.12%, 60.68% and 67.01% of the EOs from the leaves, flower buds and stems, respectively (Table 2). In the multivariate exploratory analysis to determine the major classes through PCA, it was seen that the EOs from the leaves and stems, and those from the flower buds and stems, were positively correlated with the classes of oxygenated and hydrocarbon sesquiterpenes, respectively (Figure 1).

Similar fractions of these classes of compounds were found by Zardeto-Sabec et al. (2020), in leaves (61.41%) and flower buds (65.25%) of *T. riparia*, with positive correlations of sesquiterpenes (both oxygenates and hydrocarbons) for EOs from both botanical structures. In a study conducted by Campbell et al. (1997) on the African continent, the EOs diverged regarding the class of compounds, such that monoterpenes (both hydrocarbons and oxygenates) comprised the majority (69.00%), thus confirming that these plants produce their secondary metabolites in accordance with their physiological needs and the conditions of the environment.

Evaluation of larvicidal activity showed that all the EOs from the three botanical structures analyzed significantly promoted mortality at concentrations of 100,000 and 50,000 µg/mL. This larvicidal action was already foreseeable, given the different substances with proven biological activity previously found in the EOs from this plant, with insecticidal (Fernandez et al., 2014; Oliveira et al., 2022). The results from the present study confirm the existence of acaricidal activity caused by EOs, which was demonstrated for the first time by Gazim et al. (2010).

Studies have correlated monoterpenes and sesquiterpenes with the biocidal action of EOs from different plants. Although the mechanism of action is not fully understood, it is already described in the literature, several monoterpenes are neurotoxic because they inhibit the enzyme acetylcholinesterase, and have the ability to interfere with the metabolic functions of arthropods (Jankowska et al., 2018; Medeiros et al., 2019; Yang et al., 2021). For example, 1,8-cineol (eucalyptol) extracted from *Lippia alba* has remarkable insecticidal activity (Lima et al., 2021). Moreover, eucalyptol extracted from the EOs of *Senecio cannabifolius* (Yang et al., 2021), *Melinis minutiflora* (Prates et al., 1998) and *Eucalyptus globulus* (Chagas et al., 2002) was found to be able to kill 100% of *R.* (*B.*) *microplus* larvae in the respective studies. Limonene extracted from the EOs of *Pilocarpus spicatus* was found to have intense repellent activity against *R.* (*B.*) *microplus* (Nogueira et al., 2020). In a study by Gazim et al. (2011) on TrEOs, the compounds fenchone and limonene were found to be responsible for intense biocidal activity in different life stages of *R.* (*B.*) *microplus.*

High synergism with high larval mortality rates for *R.* (*B.*) *microplus* has been demonstrated in relation to combinations of carvacrol, eugenol and thymol extracted from the EOs of *Syzygium aromaticum*, *Cinnamomum zeylanicum* and *Cymbopogon citratus* (Jyoti et al., 2019). Similar studies conducted on other species of mites and even on insects have shown that thymol is 100% effective in its larvicidal activity against the species *Rhipicephalus annulatus*, at a concentration of 2.50% (Arafa et al., 2020). The high proportion of thymol in the EOs of *Lantana camara* has also been correlated with intense insecticidal activity against *Sitophilus granarius*, thus confirming that the larvicidal action of monoterpenes is efficiently expanded even to arthropods of other classes.

With regard to sesquiterpenes, Sadgrove et al. (2020) demonstrated that the compounds spathulenol and guaiol had antimicrobial and acaricidal activity. The chemical components aromadendrene, valencene and caryophyllene oxide, extracted from the leaves of *Eugenia langsdorffii*, proved to be potent acaricides that led to mortality rates above 80%, as observed using the residual contact method (Moraes et al., 2012). Another sesquiterpene, T-cadinol, isolated from the EOs of *Casearia sylvestris*, was found to induce mitochondrial impairment of *Trypanosoma cruzi*, and thus demonstrated protozoicidal effects of importance with regard to formulating future drugs for combating Chagas disease (Santos et al., 2021). Because of this high biocidal power seen in relation to several groups of parasites, as confirmed through scientific evidence in the literature, it can be presumed that the larvicidal action seen in the present study was strongly associated with the large amounts of monoterpenes (both hydrocarbons and oxygenates) (17.42%, 17.90% and 12.30%) and sesquiterpenes (both hydrocarbons and oxygenates) (61.12%,

60.68% and 67.01%) that were present in the EOs of the leaves, flower buds and stems of *T. riparia*, respectively (Table 2).

Based on the larval mortality rate results presented in Table 3, progress was made in conducting other laboratory tests (*in vitro* and *ex situ*) to determine the $LC_{50.0}$ and $LC_{99.9}$, along with the product efficiency (PE) in relation to the larvae of *R.* (*B.*) *microplus* (Tables 4-5). The LCs were determined through Probit analyses (Table 4), and the botanical structure that proved to be the source of EOs of highest efficiency and greatest larvicidal power was the leaves, from which the concentration of EOs required was the lowest ($LC_{99.9}$ = 83.53 µg/mL). In a study conducted by Gazim et al. (2011) using EOs obtained from leaves, but harvested in summer, the $LC_{99.9}$ was 11.38 (g/mL), a LC higher than that of the present study, thus demonstrating lower biocidal effect.

These disparate results can be explained in terms of interference from environmental variations, given that synthesis of secondary metabolites in plants is quantitatively and qualitatively affected by differences in climatic conditions (Hartmann, 1996; Khakdan et al., 2021), biotic and abiotic factors (Gobbo-Neto & Lopes, 2007), geographical locations and edaphic conditions (Swamy et al., 2016) and EO extraction and storage methods (Nea et al., 2020). According to Gazim et al. (2010), the species *T. riparia* showed high chemical variability of its EOs between the different seasons of the year. This was confirmed by Cardoso et al. (2015), who observed seasonality effects relating to the concentrations of TrEO compounds.

After defining $LC_{99.9}$, the larvicidal activity was tested under semi-natural conditions (*ex situ*), to verify the chemical stability of the formula when applied in the field under uncontrolled conditions. Twenty-four hours after application of EOs from the leaves ($LC_{99.9}$ = 83.53 µg/mL), flower buds ($LC_{99.9}$ = 91.91 µg/mL) and stems ($LC_{99.9}$ = 85.42 µg/mL), more than 95% of the larvae had died. This demonstrated that the TrEOs has a significant larvicidal effect, and that there were no significant differences between the treatments (Table 5). These results also fell within the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP), which recommend that effectiveness > 95% is required, for commercial acaricides to be described as good quality (Holdsworth et al., 2006).

The high mortality rate presented in this study indicates that the TrEOs from the three botanical structures (leaves, flower buds and stems) showed chemical stability with regard to the formulation components (emulsifiers and fixing agents). No oxidation or hydrolysis reactions occurred in these biomolecules due to abiotic factors (light, humidity, O_2 and temperature).

The PE results were extremely satisfactory. All the TrEOs tested showed excellent efficacy: PE = 98.25%, 98.48% and 98.14% for EOs from the leaves, flower buds and stems, respectively. The commercial product used as the PC presented PE of 100%, without any significant differences between the treatments, which proved that the TrEOs were reaching their maximum efficiency of larvicidal activity against the species *R.* (*B.*) *microplus* under semi-natural laboratory conditions (Table 5). The PE of the TrEOs was higher than 95%, which is the minimum percentage effectiveness required by the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), for new registrations of acaricides in Brazil (Brasil, 1997). Thus, the TrEOs of the present study can form excellent prospection sources for synthesis of biomolecules for new chemical formulations of acaricides.

Comparison of the present results with data from similar studies in the literature that used other EOs to combat *R.* (*B.*) *microplus* larvae showed that the TrEOs tested in the present study had better PE, with potential for biocidal activity. Medeiros et al. (2019) studied the EOs of *Eugenia pyriformis* and found one EO with PE = 72.20% and $LC_{99.9}$ = 24.60 mg/mL. Their results were considered low because of the high LC. Araújo et al. (2015) studied thymol, a monoterpene present in the EOs of several plant species, and obtained very similar results, with EP = 99.87% but at a concentration of 20.0 mg/mL, i.e., with a LC that was much higher than that of the present study.

Approximately 95% of the ticks on any farm are found in the pastures in the free-living form (Garcia et al., 2019). Moreover, all treatments in the present study showed active high performance, such that they acted efficiently in the initial stages of the parasite cycle, both under laboratory conditions (*in vitro*) and under semi-natural conditions (*ex situ*). In addition, the life stage of ticks is an important factor that needs to be considered in order to achieve success in strategic control (Silva et al., 2020). In this light, it can be asserted that TrEOs constitute alternatives that in the future may help in combating cattle ticks with minimal environmental impact, thereby improving on the existing protocols for treatments at this stage of the cycle of this ectoparasite.

Conclusion

From the results presented in the tests (*in vitro* and *ex situ*), it was possible to conclude that the *T. riparia* essential oils EOs were effective against the larvae of *R.* (*B.*) *microplus*, thus showing that this plant has bioactive molecules

with significant larvicidal action. Further studies are needed in order to demonstrate any potential toxicity of EOs towards non-target mammals and other organisms, so as to validate the safety of use of these EOs with regard to the environment and health. Subsequently, the biocidal molecules found in TrEOs could possibly be incorporated into economically viable commercial products for strategic control of cattle ticks.

Acknowledgements

The authors thank Universidade Paranaense, Universidade do Estado do Amazonas, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Araucária for the support and the fellowship.

Ethics declaration

The proposed protocol did not use biological samples from animals belonging to the phylum Chordata and has the CEUA exemption from Universidade Paranaense - UNIPAR.

Conflict of interest

The authors declare that there are no conflicts of interest.

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