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Evaluation of Essential Oils from the Brazilian Species *Baccharis trimera* (Less.) DC. and *Mimosa verrucosa* Benth. against *Ctenocephalides felis felis* Bouché

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HIGHLIGHTS

- Essential oils rich in terpene compounds.
- *Baccharis trimera* has pulicidal potential against adult fleas
- *Mimosa verrucosa* has pulicidal potential against larvae and adult fleas.
- Essential oils have repellent activity.

Abstract: Essential oils (EO) have the potential to control ectoparasites, being an alternative to the use of synthetic products. The essential oils of *Baccharis trimera* and *Mimosa verrucosa* were evaluated for lethality, residual efficacy, and repellence against the ectoparasite *Ctenocephalides felis felis* Bouché, and toxicity was using *Saccharomyces cerevisiae* eukaryote model. EOs were obtained by steam distillation and characterized by GC/FID/MS. Mortality at different immature stages and among adult fleas was measured through *in vitro* filter paper tests at different concentrations of EOs, as well as residual effect and repellent activity. The major compounds for *B. trimera* were carquejyl acetate (33.0%), carquejol (7.07%), and palustrol

(6.99%), and for *M. verrucosa*, β -pinene (14.2%), (*E*)-caryophyllene (13.8%), and α -pinene (10.6%). Low toxicity was recorded for *B. trimera* and *M. verrucosa* EOs at 200 $\mu\text{g.mL}^{-1}$. EOs recorded 100% efficacy against adult fleas at 800 $\mu\text{g.cm}^{-2}$ ($\text{LC}_{50} = 369.22 \mu\text{g.cm}^{-2}$) and residual effect for three days, *B. trimera* EO promoted 46.7% of mortality on the 3rd day of the exposition, and *M. verrucosa* EO 20% of mortality on the 3rd day of the exposition. The repellent activity was presented in the concentration of 40000 $\mu\text{g.mL}^{-1}$ for *B. trimera* EO (80% at 12h) and *M. verrucosa* EO (75% at 6h). The results show great potential for developing alternative products from the essentials of *B. trimera* and *M. verrucosa* in control against *C. felis felis*, which show low toxicity and high activity.

Keywords: volatile oil; biocontrol; biopesticide; fleas; toxicity.

INTRODUCTION

Ctenocephalides felis felis is the most prevalent flea subspecies parasitizing companion animals worldwide [1]. Infestations by *C. felis felis* can be transmitted to humans by contact with dogs and cats or with infested environments, which can cause discomfort and allergic reactions [2].

Chemical synthetic formulations are traditionally employed for controlling fleas. These formulations raise several issues related to environmental impact and develop resistance to ectoparasites, reducing the products' effectiveness [3, 4]. Organic plant-derived compounds represent alternative controlling agents [5, 6, 7]. Plants can produce a broad range of secondary metabolites; terpenoids have well-established insecticidal activity [8, 9].

Essential oils are a complex mixture of compounds, and their chemical composition is mainly based on terpenes and phenylpropanoids [10]. They have shown to be very promising concerning their biopesticide activity due to the different bioactive compounds in their composition. The largest body of research describes their activity against mosquitoes, ticks, and fleas [11, 12]. Interest has been directed to finding new formulations for veterinary use and new active molecules or even prototypes for developing new drugs that are more effective and safer for veterinary medicine [13]. Using effective natural products could be an excellent alternative to synthetic compounds to minimize the negative impact on human health and the environment [14].

Baccharis trimera (Less.) DC. known as carqueja (Asteraceae), native to the southern region and *Mimosa verrucosa* Benth. known as jurema-branca (Fabaceae), native to the caatinga biome and the northeast region, are Brazilian plants used in folk medicine [15,16]. *B. trimera* showed carquejyl acetate as a major compound, while *M. verrucosa* is studied for the first time in the present article. Moreover, few papers published with Brazilian plants show the chemical composition of essential oil and pulicidal activity.

The present study aimed to evaluate the pulicidal and repellent activity of the essential oils (EOs) of *B. trimera* and *M. verrucosa* against the different immature stages and adults of the flea *C. felis felis*, characterize their residual effect, and evaluate the toxicity of these oils in a eukaryotic organism mimicking the cells of higher mammals.

MATERIAL AND METHODS

Essential Oils and their Chemical Characterization

Essential oils from *B. trimera*, known as carqueja, from all plant parts, and *M. verrucosa*, known as jurema-branca, from leaves and twigs, were purchased commercially (Laszlo®). Both essential oils were obtained by steam distillation, protected from light, and stored at -20 °C until all the analyses were performed. To separate, detect, and quantify the constituents, 1 μL of essential oils samples (10 $\mu\text{L.mL}^{-1}$), in the defined times, were injected into the gas chromatography (GC). A Hewlett-Packard 5890 Series II (Palo Alto, USA), equipped with flame ionization detection and a split/splitless injector in a split ratio 1:20, was used to separate and detect the constituents in the essential oil. The substances were separated with a fused silica capillary column, similar DB5 with 30 m $\times$ 0.25 mm (i.d.) $\times$ 0.25 μm (film thickness). Helium was used as the carrier gas at a 1.0 mL.min^{-1} flow rate. The column temperature was programmed as follows: 60 °C for 2 min followed by heating at 5 °C min^{-1} to 110 °C, followed by heating at 3 °C min^{-1} to 150 °C and finally followed by heating at 15 °C min^{-1} until 290 °C and holding constant for 15 min. The injector temperature was 220 °C and the detector temperature was 290 °C. To separate and identify the substances, 1.0 μL of essential oils samples (10 $\mu\text{L.mL}^{-1}$), in the defined times, were injected in the gas chromatograph coupled to a mass spectrometer (GC-MS) QP-2010 Plus (Shimadzu, Japan). The flow of the helium gas carrier, the capillary column, and the temperature conditions for the GC-MS analysis were the same as those described for the GC. The

temperature of the injector was 220 °C and the temperature of the interface was 250 °C. Mass spectra were obtained with a quadrupole detector operating at 70 eV, with a 40–400 m/z mass range and scanning rate equal to 0.5 scan s⁻¹. The identification of volatile compounds in essential oil has been based on Linear Retention Indices (LRI) and mass spectra of the samples, compared with authentic standards injected under the same conditions, with the NIST database (2008) and the index Kovats, IK [17]. The LRI was calculated based on the co-injection of alkanes series [18].

Biological tests

The experiments followed the standards established by the Ethics Committee for Animal Use of the Veterinary Institute (CEUA/IV nº 4313110419). Fleas (eggs, larvae, pupae, and adults) were obtained from a colony of *C. felis* maintained in the Experimental Chemotherapy Laboratory in Veterinary Parasitology (LQEPV) at the Federal Rural University of Rio de Janeiro (UFRRJ).

In vitro activity of essential oils against *Ctenocephalides felis felis*

Bioassays for evaluating the mortality of the fleas against the essential oils were performed using the filter paper impregnation method [18], where a stock solution was prepared at 200 mg.mL⁻¹ of essential oils, and from this, 10 serial dilutions were performed 1:2, made in acetone, an innocuous solvent used as a negative control capable of solubilizing the essential oil, with a ranging concentration from 1978.1 µg.mL⁻¹ to 49.5 µg.mL⁻¹. Fipronil (technical-grade active ingredient fipronil (99.2%)) was provided by CEVA® (Paulínia, BR) and used at 400 µg.cm⁻² as a positive control.

Six replications were performed for each concentration, with filter paper strips (10 cm²) for adult fleas and filter paper discs (23.76 cm²) for immature stages. The strips were impregnated with 200 µL, and the discs with 470 µL of their respective dilutions, so the final concentrations for *B. trimera* and *M. verrucosa* were 50 to 2000 µg.cm⁻².

After impregnation and drying, the strips were placed in test tubes containing 10 non-fed adults of *C. felis felis*, which were then sealed with non-woven fabric and elastic. Ten eggs, larvae, or pupae of *C. felis felis* were added to their respective impregnated discs and then sealed in the petri dish. The tubes and discs were kept in an acclimatized chamber at 27 ± 1°C, 75 ± 10% RH. Using a stereoscopic microscope (Wild Heerbrugg, Switzerland, M5-52706), the results evaluated the mean number of live fleas per concentration. Ovicidal tests were evaluated after 72 h, the larvicidal test after 24 and 48 h, the pupicidal test after 15 days, and the test for adult fleas was evaluated after 24 and 48 h.

Insecticidal activity was evaluated by impregnating filter paper with adult fleas, which were considered alive even though the fleas had less movement. So, it was determined the residual effect of the essential oil [19] obtained results at a concentration of 40000 for *B. trimera* and 100000 µg.mL⁻¹ for *M. verrucosa*. The evaluation was done for 6 days each 24 h; fleas alive were counted, and new ones were added. The impregnated tapes were maintained to evaluate the durability of the insecticidal activity of the essential oils in only one application.

Bioassays of the repellent activity against fleas were carried out to determine the fleas' resistance using Diethyltoluamide (DEET) at 40000 µg.mL⁻¹ as a positive control, using the filter paper impregnation method [19]. Bioassays for mortality were also carried out, with essential oil concentrations of 5000, 20000, and 40000 µg.mL⁻¹. These tests were evaluated after different times, varying from 1 to 48 h.

The evaluation of the *in vitro* efficacy of each concentration followed the formula developed by Abbott, used to calculate the percent efficacy [20]:

$$\frac{\text{mean number of live fleas in the control group} - \text{mean number of live fleas in the treated group}}{\text{mean number of live fleas in the control group}} \times 100$$

The assessment of lethal concentrations that killed 50% and 90% of the treated population, LC₅₀ and LC₉₀, respectively, was established by analysis in the RStudio Team software program (2020, RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA, USA).

Microorganism and cell culture conditions

Saccharomyces cerevisiae strain code BY4741 with genotype *MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0* was acquired from Euroscarf, headquartered in Frankfurt, Germany. For all bioassays, an inoculum was made from fresh subcultures in a 2% YPD (Kasvi, Brazil) medium liquid (2% glucose, 2% peptone, and 1% yeast extract). Cell proliferation was carried out overnight in a refrigerated incubator at 160 rpm and 28 °C.

Cells were grown to the stationary phase, and an aliquot of this cell suspension was collected and used for each subsequent assay.

Evaluation of the toxicity of essential oil in a eukaryote model of *Saccharomyces cerevisiae*

The toxicity of essential oils was preliminarily analyzed using the qualitative method (resazurin, Sigma Brazil) and then using two quantitative assays (cell viability and growth curves) [21]. The analysis with the resazurin reagent is based on a screening using different concentrations of the two essential oils in contact for 24 h with 50 μL of cell suspension in a 96-well plate. Then, 10 μL of 0.01% resazurin in water was added to each well, where a blue stain revealed poor cellular metabolism and a pink stain revealed active cellular metabolism.

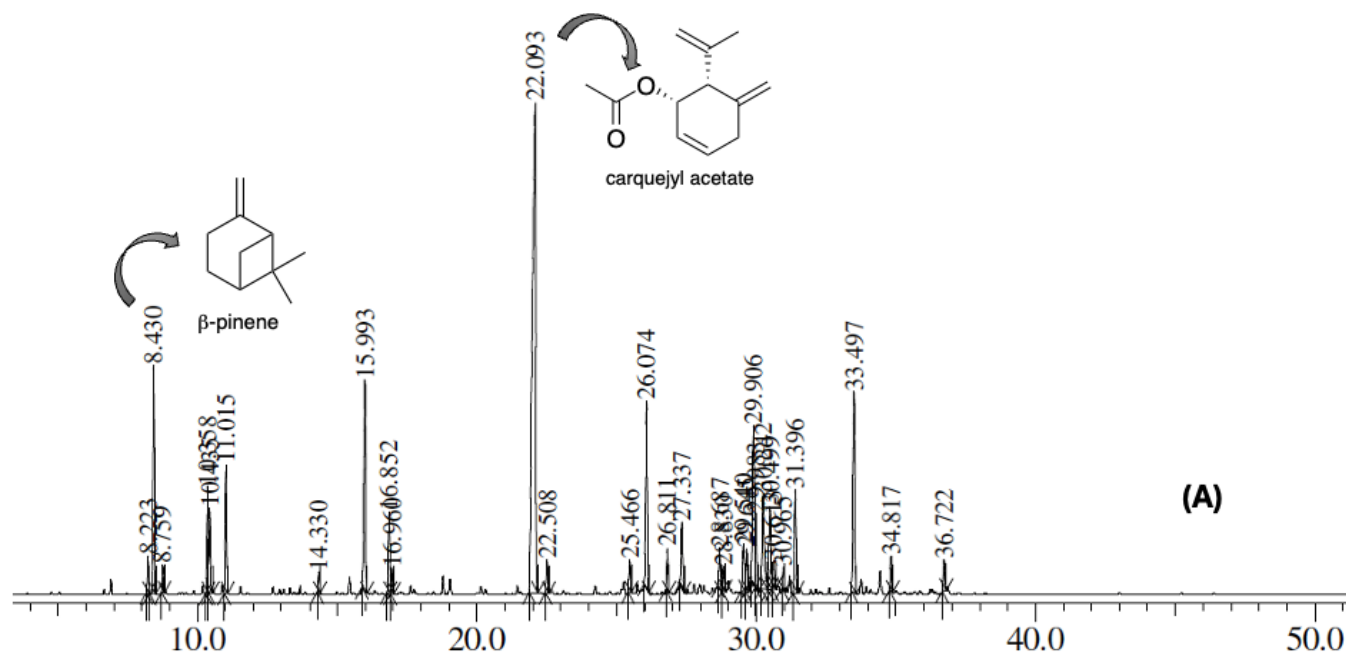
In the analysis of cytotoxicity by cell viability, an aliquot of the cell suspension (10 mg of cells per 10 mL of YPD medium) was incubated with *B. trimera* and *M. verrucosa* oils, both at a concentration of $0.2 \text{ mg} \cdot \text{mL}^{-1}$, for 1 h at 28 °C and 160 rpm. After this period, an aliquot (40 μg) of this suspension was diluted and placed in Petri dishes with 2% solid YPD medium (agar). The same aliquot was placed on plates with YPGly medium (4% glycerol replacing glucose). After 48 h in a BOD (Solab, Brazil) oven at 28 °C, the colonies were counted, and the results were compared with the control assay (cells only) and with a yeast toxicity control (H_2O_2 - 1.0 mM). Cell viability in YPD and YPGly (Kasvi, Brazil) medium was expressed in colony-forming units.

The second toxicity analysis relates to the generation of daughter cells during exposure to oils for 24 h (time-dependent). The cells were incubated with *B. trimera* and *M. verrucosa* EOs ($0.2 \text{ mg} \cdot \text{mL}^{-1}$), and the absorbance was measured in a spectrophotometer (Biospectro, Brazil) at 570 nm during 0, 2, 6, and 24 h.

RESULTS

Chemical characterization of essential oils

The analysis of essential oils led to the identification of thirty compounds for *B. trimera* and thirty for *M. verrucosa* (Figure 1 and Table 1).



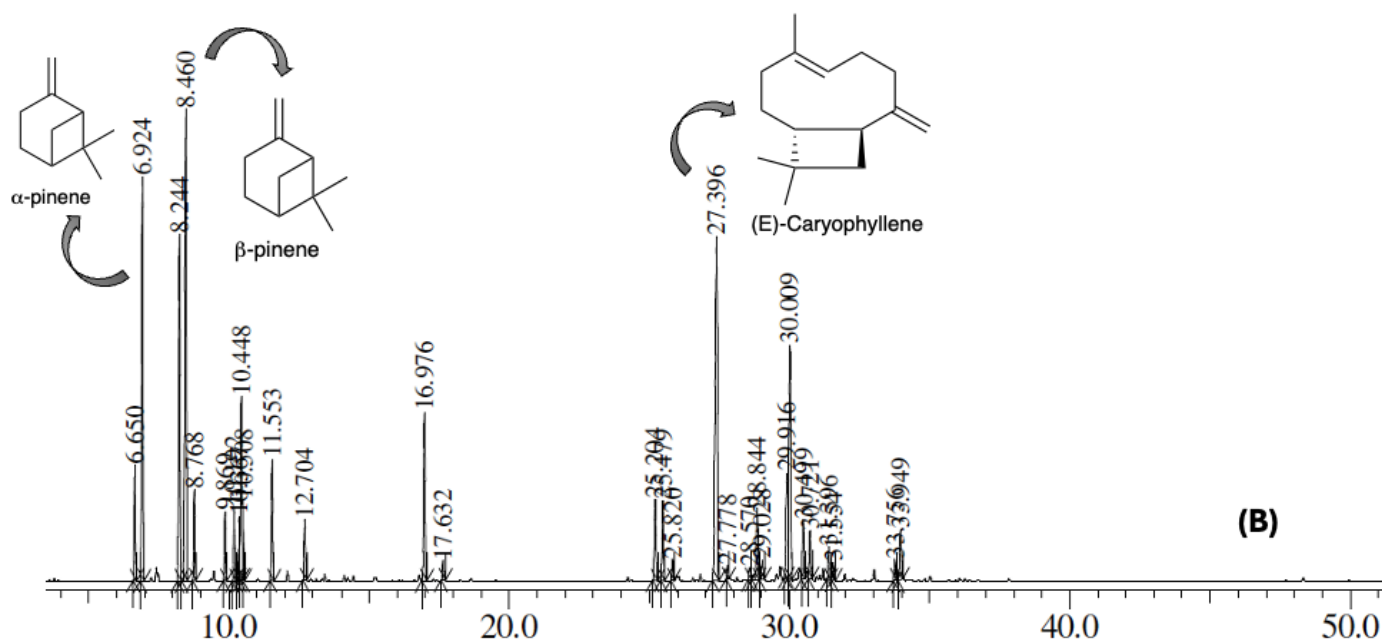


Figure 1. Chromatograms of *Baccharis trimera* (A) and *Mimosa verrucosa* (B), essential oil obtained from a fused silica capillary column similar to DB-5 (30 m x 0.25 mm x 0.25 μ m). The major compounds and the respective retention times are shown.

Table 1. Chemical composition of *Baccharis trimera* and *Mimosa verrucosa* essential oil.

Compounds	LRI ^C	LRI ^L	EOBT%	EOMV%
Monoterpenes hydrocarbons				
α -thujene	927	924	-	2.31
α -pinene	935	932	-	10.66
Sabinene	974	969	0.76	9.54
β -pinene	980	974	5.54	14.17
Myrcene	990	988	0.53	1.87
α -terpinene	1019	1014	-	1.53
<i>p</i> -cymene	1027	1020	-	1.64
Limonene	1031	1024	2.64	1.57
β -phellandrene	1033	1025	1.96	4.94
1,8-cineole	1034	1026	-	1.29
(<i>E</i>)- β -ocimene	1047	1044	3.02	-
γ -terpinene	1060	1054	-	2.93
<i>p</i> -mentha-2,4(8)-diene	1087	1085	-	1.47
Oxygenated monoterpenes				
Isophorone	1125	1118	0.49	-
Carquejol	1162	1162	7.07	-
n.i.	1181		2.21	-
Terpinen-4-ol	1184	1174	0.64	4.77
α -terpineol	1199	1186	-	0.59
Carquejyl acetate	1299	1298	33.04	-
Sesquiterpenes hydrocarbons				
n.i.	1308		0.84	-
α -ylangene	1370	1373	-	2.54
α -copaene	1376	1374	0.84	2.27
β -bourbonene	1385	1387	-	0.56
β -elemene	1391	1389	5.88	-
(<i>Z</i>)-caryophyllene	1408	1408	1.16	-
(<i>E</i>)-caryophyllene	1421	1417	1.82	13.84
β -copaene	1431	1430	-	0.35
(<i>E</i>)-muurola-3,5-diene	1451	1451	-	0.38
(<i>E</i>)- β -farnesene	1453	1454	0.99	-

Cont. Table 1

α -humulene	1457	1452	0.66	2.25
α -neocallitropsene	1477	1474	1.24	-
Germacrene D	1483	1480	1.69	3.37
γ -himachalene	1485	1481	-	8.09
α -zingiberene	1491	1493	3.74	-
Bicyclogermacrene	1497	1500	2.70	1.68
α -muurolene	1500	1500	0.76	-
β -bisabolene	1503	1505	-	1.36
Germacrene A	1509	1508	0.54	-
δ -cadinene	1520	1522	3.07	0.92
(Z)-calamenene	1524	1528	-	0.50
Oxygenated sesquiterpenes				
Palustrol	1573	1567	6.99	-
Spathulenol	1580	1577	-	0.59
Caryophyllene oxide	1585	1582	-	1.50
Viridiflorol	1607	1592	0.97	-
β -eudesmol	1659	1649	0.94	-
Total			92.73	99.48

AI^C – Arithmetic Index calculated; AI^L – Arithmetic Index literary; n.i. – not identified; EOBT – Essential Oil *Baccharis trimera*; EOMV – Essential Oil of *Mimosa verrucosa* [22].

Flea mortality at different stages, residual effect, and repellent activity

B. trimera and *M. verrucosa* EO showed activity against immature stages and adults of *C. felis felis* in the concentration range tested. The lethal concentrations at 50% and 90% were calculated from them, as shown in Table 2.

Table 2. Lethal concentrations at 50% and 90% from *Baccharis trimera* and *Mimosa verrucosa* essential oil against adults and immature stages of *Ctenocephalides felis felis*.

Stage	100% activity ($\mu\text{g.cm}^{-2}$)	LC ₅₀ ($\mu\text{g.cm}^{-2}$)	LC ₉₀ ($\mu\text{g.cm}^{-2}$)	Slope	R ²	Chi-squared	P
<i>Baccharis trimera</i> Essential Oil							
Egg (72 h)	1582.5	309.52	1391.85	1.96	0.984	16.835	0.995
Larvae (24 h)	59.3	34.56	44.93	11.25	0.912	3.400	0.361
Larvae (48 h)	59.3	34.57	44.93	11.25	0.912	3.400	0.361
Pupae (15 days)	1582.5	324.13	1160.35	1.87	0.977	13.140	0.959
Adult (24 h)	800	369.22	678.13	2.58	0.979	81.770	1.000
Adult (48 h)	800	369.22	678.13	2.58	0.979	81.770	1.000
<i>Mimosa verrucosa</i> Essential Oil							
Egg (72 h)	791.2	194.5	563.08	2.19	0.960	7.900	0.900
Larvae (24 h)	1186.9	266.29	731.34	2.92	0.925	8.933	0.823
Larvae (48 h)	791.2	240.54	551.09	3.56	0.913	8.074	0.767
Pupae (15 days)	-	-	-	-	-	-	-
Adult (24 h)	800	369.22	678.13	2.58	0.979	81.770	1.000
Adult (48 h)	800	369.22	678.13	2.58	0.979	81.770	1.000

B. trimera EO showed activity against all stages evaluated, demonstrating greater larvicidal (LC₉₀ = 44.93 $\mu\text{g.cm}^{-2}$) activity, followed by adulticidal (LC₉₀ = 678.13 $\mu\text{g.cm}^{-2}$), pupicidal (LC₉₀ = 1160.35 $\mu\text{g.cm}^{-2}$) and ovicidal (LC₉₀ = 1391.85 $\mu\text{g.cm}^{-2}$). *M. verrucosa* EO, on the other hand, showed greater ovicidal (LC₉₀ = 563.08 $\mu\text{g.cm}^{-2}$) activity, followed by adulticidal (LC₉₀ = 678.13 $\mu\text{g.cm}^{-2}$) and larvicidal (LC₉₀ = 731.34 $\mu\text{g.cm}^{-2}$) and did not demonstrate significant pupicidal activity in the concentration range evaluated.

B. trimera and *M. verrucosa* EOs presented adulticidal activity with similar potency, as they presented 100% mortality at the same concentration (800 $\mu\text{g.cm}^{-2}$). However, regarding activity against immature stages, *B. trimera* and *M. verrucosa* EOs showed different results. In terms of larvicidal activity, *B. trimera* EO presented 20-fold potency than *M. verrucosa* EO since the concentrations needed to achieve 100% mortality against larvae for *B. trimera* EO and *M. verrucosa* EO were 59.5 $\mu\text{g.cm}^{-2}$ and 1186.9 $\mu\text{g.cm}^{-2}$, respectively. The ovicidal activity was more pronounced for *M. verrucosa* EO, demonstrating 2.5-fold

potency than *B. trimera* EO, since the concentrations necessary to achieve 100% mortality against eggs for *M. verrucosa* EO and *B. trimera* EO were 791.2 $\mu\text{g.cm}^{-2}$ and 1582.5 $\mu\text{g.cm}^{-2}$, respectively.

The residual effect of insecticidal activity for both essential oils was three days. *B. trimera* EO caused 83.3% of mortality on the 2nd day of exposition, decreasing to 46.7% on the 3rd day of analysis. On the 4th day, it was still capable of causing 18.3% mortality of the fleas, and on the 5th day, there was no activity. *B. trimera* EO presented a lower concentration, capable of causing approximately 80% of mortality, and its effectiveness remained more than *M. verrucosa* EO. On the 2nd day of the exposition, the pulicidal activity of *M. verrucosa* EO decreased by approximately 40% of mortality, dropping by 58.3%, on the 3rd day, 20% of mortality and no activity on the 4th day of the exposition, as shown in Table 3.

Table 3. The residual effect from the concentration of 40000 $\mu\text{g.mL}^{-1}$ *Baccharis trimera* and *Mimosa verrucosa* essential oil against *Ctenocephalides felis felis* adult stage.

Time (days)	Total Live Fleas	Total Dead Fleas	Total Mortality (%)
<i>Baccharis trimera</i> Essential Oil			
1	0	60	100.0
2	10	50	83.3
3	32	28	46.7
4	49	11	18.3
5	60	0	0.0
<i>Mimosa verrucosa</i> Essential Oil			
1	0	60	100.0
2	25	35	58.3
3	48	12	20.0
4	60	0	0.0
5	60	0	0.0

Essential oils of *B. trimera* and *M. verrucosa* showed repellent activity against *C. felis felis* in all concentrations tested. Negative control (acetone) was ineffective, and positive control (DEET) presented a satisfactory variation of effectiveness over time under exposed concentrations. The best efficiency results were found for *B. trimera* EO in concentrations of 20000 and 40000 $\mu\text{g.mL}^{-1}$; at higher concentrations, it maintained repellence in the range of 80% for 12h and reached 50% in 24 h. Even for *M. verrucosa*, EO was the concentration of 40000 $\mu\text{g.mL}^{-1}$ effective at the first hour of evaluation reached 83.1%, in 6 h reached 75%, and in 12h, 7.8% of repellence, as shown in Table 4.

Table 4. Percentage of repellence from *Baccharis trimera* and *Mimosa verrucosa* essential oil against adult stage of *Ctenocephalides felis felis*.

Essential Oil	$\mu\text{g.mL}^{-1}$	$\mu\text{g.cm}^{-2}$	1 h	3 h	6 h	12 h	24 h	48 h
<i>Mimosa verrucosa</i>	5000	100.0	50.0 ^{a, c, e}	46.2 ^{a, c, e}	28.6 ^{a, c, e}	18.2 ^{a, c, e}	0.0 ^{a, c, e}	0.0 ^{a, c, e}
	20000	400.0	69.5 ^{a, c, e}	57.1 ^{a, c, e}	57.1 ^{a, c, e}	18.2 ^{a, c, e}	0.0 ^{b, c, e}	0.0 ^{b, c, e}
	40000	800.0	83.1 ^{a, d, e}	86.8 ^{a, d, e}	75.0 ^{a, c, e}	37.8 ^{b, c, e}	0.0 ^{b, c, e}	0.0 ^{b, c, e}
<i>Baccharis trimera</i>	5000	100.0	37.8 ^{a, c, e}	28.6 ^{a, c, e}	12.5 ^{a, c, e}	0.0 ^{a, c, e}	0.0 ^{a, c, e}	0.0 ^{a, c, e}
	20000	400.0	84.6 ^{a, c, e}	84.6 ^{a, d, e}	75.0 ^{a, d, e}	69.6 ^{a, d, f}	28.6 ^{b, c, f}	0.0 ^{b, c, e}
	40000	800.0	88.9 ^{a, d, e}	88.9 ^{a, d, e}	80.0 ^{a, d, e}	84.6 ^{a, d, f}	50.0 ^{b, d, f}	28.6 ^{b, c, e}
DEET	5000	100.0	70.0 ^{a, c, e}	53.3 ^{a, c, e}	63.3 ^{a, c, e}	56.7 ^{a, c, e}	53.3 ^{a, c, f}	23.3 ^{a, c, e}
	20000	400.0	93.3 ^{a, c, e}	96.7 ^{a, c, f}	73.3 ^{a, c, e}	73.3 ^{a, c, f}	60.8 ^{a, c, f}	36.7 ^{a, c, e}
	40000	800.0	100.0 ^{a, c, f}	100.0 ^{a, c, f}	83.3 ^{a, c, e}	80.0 ^{a, c, f}	80.0 ^{a, c, f}	80.0 ^{a, d, f}
Placebo	- - -	- - -	6,5	0,0	0,0	6,5	0,0	0,0

Differences letter ^{a, b} statistical difference between the concentrations of the same essential oil or DEET in different time frames; ^{c, d} statistical difference of the same concentration in various time frames of the same essential oil; ^{e, f} statistical difference between different essential oils in the same concentration and same time frames.

Toxicity of essential oils

Qualitative results in the resazurin assay revealed a non-cytotoxic concentration range for yeast cells, specifically between 8000.00 and 15.563 $\mu\text{g.mL}^{-1}$ (data not shown). Based on this assay, we chose an intermediate concentration (200 $\mu\text{g.mL}^{-1}$) of this screening for a quantitative assessment of the toxicity of essential oils. Cell viability evaluates the number of viable cells, which are individual cells capable of forming colonies. Colonies were counted on YPD medium (medium rich in glucose - fermentative metabolism) and YPGly (medium rich in glycerol - respiratory metabolism).

The results of Figure 2 in YPD medium showed a low toxicity of *B. trimera* EO (176.50 ± 4.12 CFU) and *M. verrucosa* EO (171.80 ± 3.27 CFU) concerning the control (203.00 ± 6.96 CFU). The control was cells without any treatment incubated in a culture medium under the same agitation and temperature parameters. However, both essential oils showed low toxicity compared to the positive control (hydrogen peroxide) (75.00 ± 5.10 CFU). It is important to emphasize that none of the treatments with essential oils of both species at a concentration of 200 $\mu\text{g.mL}^{-1}$ showed statistical similarity with the control's cell viability level ($P < 0.05$).

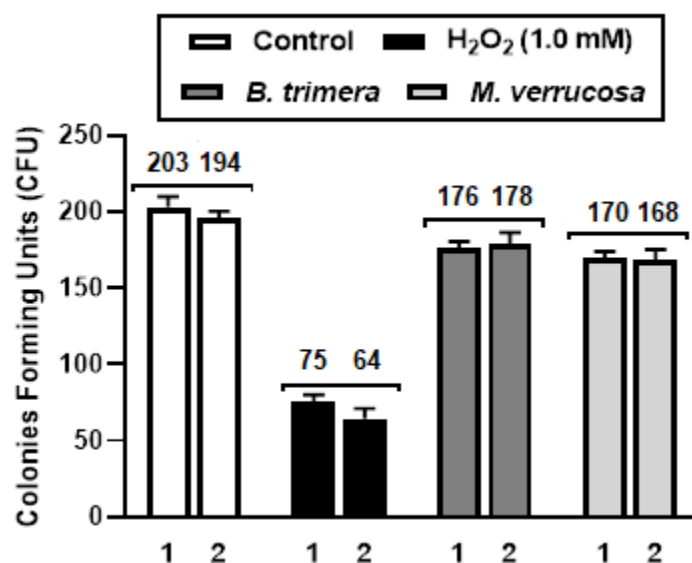


Figure 2. Colonies of *Saccharomyces cerevisiae* cells in different culture media (1-YPD and 2-YPGly) after exposure to *B. trimera* and *M. verrucosa* oils (200 $\mu\text{g.mL}^{-1}$), counted after 48 h of cultivation. *H₂O₂* (1.0 mM) was used as a toxicity standard for yeast cells. Different amounts in the number sign represent results that are statistically different.

Evaluating cell growth in contact with the essential oils over time may reveal a time-dependent growth inhibition. Figure 3 shows that both essential oils (*B. trimera* and *M. verrucosa*) did not inhibit the growth of *S. cerevisiae* cells, as they presented statistically similar curves to the control ($P < 0.05$) at all times. Hydrogen peroxide, on the other hand, proved to be toxic to cells from 2 h to 24 h.

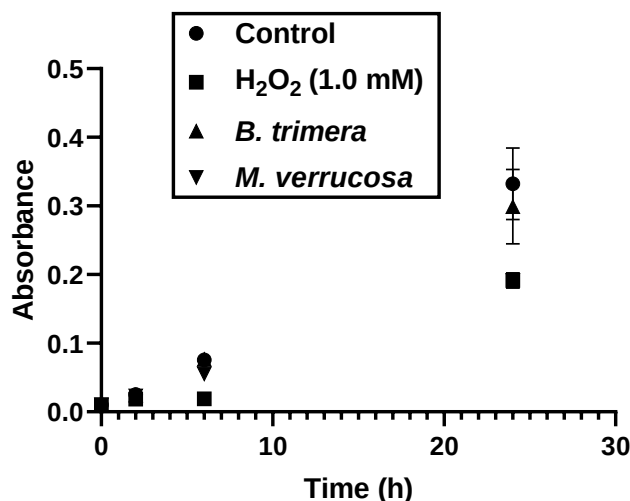


Figure 3. *Saccharomyces cerevisiae* growth curve represented by absorbance at 570 nm versus time.

DISCUSSIONS

This is the first study using the essential oils of *B. trimera* and *M. verrucosa* *in vitro*, correlating their toxicity to the mortality of animal ectoparasites. Analysis of the chemical profile of essential oils by gas chromatography revealed their complexity, enabling the identification of the numerous compounds responsible for their properties.

For *B. trimera* essential oil, 30 compounds were identified, of which carquejyl acetate (33.04%) and carquejol (7.07%) were the major. According to Suzuki and coauthors [23], carquejyl acetate acts synergistically with β -pinene, also present in the essential oil composition (5.54%).

Thirty compounds were identified in the composition of *M. verrucosa* essential oil, among which α -pinene (10.66%), sabinene (9.54%), β -pinene (14.17%), *E*-caryophyllene (13.84%), and γ -himachalene (8.09%) were the major ones. According to Suzuki and coauthors [23], β -pinene is one of the most important bioactives in the chemical composition of several essential oils and can produce an antimicrobial effect, as it has characteristics that can change the membrane of bacterial cells.

According to Avelar and coauthors [24], essential oils from the *Schinus molle* species contain monoterpenes and sesquiterpenes as the major classes. As described by Batista and coauthors [13], the essential oil of *S. molle* was 100% effective against fleas of the species *C. felis felis*. The similar chemical composition of *S. molle* and *M. verrucosa* could indicate the good efficacy against fleas found in our study.

Among the different concentrations evaluated in each stage, those that obtained 100% mortality efficiency in both essential oils were present. Only the *M. verrucosa* essential oil test against the pupae did not show satisfactory results.

Then, it is possible to state that the result for adult fleas for both essential oils analyzed, *B. trimera* and *M. verrucosa* ($LC_{50} = 369.22 \mu\text{g.cm}^{-2}$ in 24 and 48 h), corroborates the literature data, with lower lethal concentration to the mentioned EO and equivalent to the essential oil of *Schinus molle* fruits ($LC_{50} = 353.95 \mu\text{g.cm}^{-2}$), *Alpinia zerumbeti* ($LC_{50} = 553.31 \mu\text{g.cm}^{-2}$), *Laurus nobilis* ($LC_{50} = 412.09 \mu\text{g.cm}^{-2}$), *Mentha spicata* ($LC_{50} = 597.56 \mu\text{g.cm}^{-2}$) and *Cymbopogon nardus* ($LC_{50} = 597.05 \mu\text{g.cm}^{-2}$) [13, 25].

From the results for larvae fleas, it is possible to verify that a low lethality was obtained, *B. trimera* EO obtained $LC_{50} = 34.57 \mu\text{g.cm}^{-2}$ in 24 and 48 h, and the *M. verrucosa* EO obtained $LC_{50} = 266.29 \mu\text{g.cm}^{-2}$ in 24 h and $LC_{50} = 24.54 \mu\text{g.cm}^{-2}$ in 48 h. Compared to recent studies in the literature, *Illicium verum* essential oil obtained $LC_{50} = 14.4 \mu\text{g.cm}^{-2}$ in 24 h and $LC_{50} = 12.1 \mu\text{g.cm}^{-2}$ in 48 h, *Pelargonium graveolens* obtained $LC_{50} = 16.3 \mu\text{g.cm}^{-2}$ in 24 h and $LC_{50} = 20.1 \mu\text{g.cm}^{-2}$ in 48 h [12], *Alpinia zerumbeti* obtained $LC_{50} = 7.29 \mu\text{g.cm}^{-2}$ in 24 h, *Cinnamomum* obtained $LC_{50} = 0.43 \mu\text{g.cm}^{-2}$ in 24 h, *Laurus nobilis* obtained $LC_{50} = 0.52 \mu\text{g.cm}^{-2}$ in 24 h, *Ocimum gratissimum* obtained $LC_{50} = 1.21 \mu\text{g.cm}^{-2}$ in 24 h and *Cymbopogon nardus* obtained $LC_{50} = 7.32 \mu\text{g.cm}^{-2}$ in 24 h [25].

Residual effects should be highlighted in the development of new biopesticides. In literature, few studies show the essential oils' residual effect against fleas [19], even though evaluating the activity duration of the essential oils in the environment and the host is essential. This study shows that the pulicidal activity of these essential oils remained above 50% for three days in the concentration of $800 \mu\text{g.cm}^{-2}$, corresponding to 100% mortality of adult fleas against *B. trimera* EO. On the other hand, *M. verrucosa* EO in the concentration of $240 \mu\text{g.cm}^{-2}$, which corresponds to 100% of mortality against adult fleas, had a residual effect of approximately 50% after two days.

The repellent activity of the *B. trimera* EO and *M. verrucosa* EO decreased during evaluation. Over time, the impact under fleas decreases, corroborating with the positive control, as shown in Table 4. Another important observation about repellent activity is its proportional increase to the increase of applied concentration. *B. trimera* EO was more efficient under higher concentrations because its repellence varied from 88.9% to 84.6% from 1 to 12 h and, in 24 h, reached 50%. In comparison, *M. verrucosa* EO presented 83.1% of repellence, increasing its activity in 3 h, 86.8%, and 12h reached 37.8%.

S. cerevisiae is a microorganism widely used in biological assays to evaluate the toxicity of synthetic and natural substances, including essential oils [26, 27]. Its great genetic orthology with higher mammalian cells producing proteins with identical functions in both cells characterizes this yeast as a eukaryotic model aiming at preliminary tests on the biological activity of exogenous substances [26].

Cell viability in the YPD medium shows that the cells placed in contact with the EOs obtained statistically similar results and very close results to the control, revealing low toxicity. However, the stressor agent used as a parameter showed high toxicity to *S. cerevisiae* cells.

The study by [27] showed a non-toxic effect, but a trophic effect was inhibited by the action of *B. trimera* essential oil. Recent studies on the chemical properties of medicinal plants, associating the extract of *B. trimera* and the presence of terpene compounds, demonstrate that toxicopharmacological activities are rarely found in bone marrow cells of Wistar rats [28]. Among the few studies suggest that despite the hemolytic

action found for the *B. trimera* extract, the biological efficacy at the concentrations tested does not reveal cytotoxicity *in vitro* [29].

Recent studies on some terpene compounds found in the species reinforce the assertion. The chemical compound 1,8-cineol, present in the species *M. verrucosa*, has a repellent and insecticide action for *Periplaneta americana*, in addition to having a larvicidal and ovipositive repellence for mosquitoes [30]. The predominance of the sesquiterpene compounds, caryophyllene, germacrene, spathulenol, β -elemene, and α -humulene, has demonstrated several biological activities by inhibiting cell proliferation [31, 32].

There was no difference in toxicity between the species *B. trimera* and *M. verrucosa* in the exposure of cells to essential oils during 24 h (growth curves). Although the essential oil from *B. trimera* has carquejyl acetate and β -pinene as the major compounds and the essential oil from *M. verrucosa* has β -pinene as the major compound, this was not enough for differentiating toxicity results.

CONCLUSION

The objectives of the study were achieved since both oils were chemically identified, demonstrating that they are rich in terpene compounds. *B. trimera* essential oil was demonstrated to have pulicidal potential against adult fleas, and *M. verrucosa* essential oil showed to have pulicidal potential under the larvae and adult fleas, presenting durability of residual effect and efficiency of repellence activity, with both presenting low toxicity. These results are useful for following up on studies with other authors, aiming at developing products to be used as an alternative in the control of ectoparasites.

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