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A N I N T E R N A T I O N A L J O U R N A L

Influence of Essential Oil Fractionation by Vacuum Distillation on Acaricidal Activity Against the Cattle Tick

Fernando Cidade Torres[1,2], Aline Machado Lucas[1], Vera Lucia Sardá Ribeiro[2], João Ricardo Martins[3], Gilsane von Poser[4], Maria Silvia Guala[5], Heriberto Victor Elder[5] and Eduardo Cassel[1*]

[1]Laboratório de Operações Unitárias; Faculdade de Engenharia; Pontifícia Universidade Católica do Rio Grande do Sul; Av. Ipiranga, 6681; 90619-900; Porto Alegre - RS - Brasil. [2]Laboratório de Acarologia e Entomologia; Faculdade de Veterinária; Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9090; 91540-000; Porto Alegre - RS - Brasil. [3]Instituto de Pesquisas Veterinárias Desidério Finamor; Estrada do Conde s/n; Guaíba - RS-– Brasil. [4] Programa de Pós-Graduação em Ciências Farmacêuticas; Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752; 90610-000; Porto Alegre - RS - Brasil. [5]Facultad de Ingeniería Química; Universidad Nacional del Litoral; Santa Fe - Argentine

ABSTRACT

The aim of this work was to study the influence of essential oil fractionation on acaricidal activity against the cattle tick Rhipicephalus *(*Boophilus*)* microplus*. The citronella (*Cymbopogon winterianus *J.) and pepper tree (*Schinus molle *L.) essential oils were fractionated by vacuum distillation yielding fractions that were analyzed by the GC/MS. Laboratory tests were carried out to determine the effect of the total essential oil and fractions on larvae of the cattle tick* R. *(*B.*)* microplus*. The fractions 04 and 05 of the* C. winterianus *essential oil were the most active showing LC_{50} values of 1.20 and 1.34 μL/mL, respectively. The LC_{50} of the total oil was 3.30 μL/mL while the effect of the fractions 01, 02 and 03 was less pronounced, with LC_{50} values of 4.37, 4.24 and 3.49 μL/mL, respectively. The fraction 03 of the* S. molle *essential oil was the most active showing LC_{50} value of 8.80 μL/mL while the fractions 01 and 02 did not show toxic effects on the larvae.*

Key words: *Cymbopogon winterianus* J., *Schinus molle* L., acaricidal activity, vacuum distillation, *Rhipicephalus* (*Boophilus*) *microplus*

INTRODUCTION

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* represents a major problem for the cattle production in several regions of the world. It causes blood loss, reduction in both weight gain and milk production. It also serves as a vector for certain diseases such as bovine babesiosis, a cattle tick-borne disease caused by the haemoprotozoan parasites of the genus *Babesia*, and anaplasmosis, caused by obligate intracellular bacteria of the genus *Anaplasma* that parasitize erythrocytes and monocytes of higher vertebrates, mostly ruminants. In addition, the parasite causes damage to skin of the cattle directly affecting the value of hides for leather manufacture (García-García et al. 1999; Ducornez et al. 2005). The economical losses world-over were estimated as $7 billion (Castro-Janer et al. 2009) due to this.

The most extensively used methods for tick control involve the application of acaricides such as pyrethroids and formamidine, both in the

*Author for correspondence: cassel@pucrs.br

environment and directly on the animal skin. However, the indiscriminate use of these substances leads to increase in acaricide resistance and environmental contamination (Chagas et al. 2002; Chagas 2004). Therefore, effective and easily accepted methods of tick control are extremely necessary (Cordovés 1997).

Natural products can be considered a valuable alternative. A large number of plants and entomopathogenic agents have been reported to have acaricidal effect against the diverse stages of several species of ticks such as *Ixodes scapularis* and *R.* (*B.*) *microplus* (Borges et al. 2003; Freitas-Ribeiro et al. 2005; Dietrich et al. 2006; Ribeiro et al. 2007; Fernandes et al. 2008; Ribeiro et al. 2008). Among them, essential oils components are known to possess repellent, chemosterilant, antifeeding and biocidal activities against different acarus, showing promissory potential as products for tick control, since some of them are selective and have little or no harmful effects on non-target organisms (Facey et al. 2005; Panella et al. 2005; Dietrich et al. 2006; Tunon et al. 2006; Apel et al. 2009).

The genus *Cymbopogon*, family Poaceae, comprises a group of about 40 tropical aromatic grasses. The essential oils from several species of this genus have demonstrated insecticidal and repellent activity (Kim et al. 2004; Ketoh et al. 2006; Samarasekera et al. 2006). The citronella (*Cymbopogon winterianus* J.) essential oil presents repellent properties and represents a significant parcel of the essential oil national production being used in sprays, soaps, candles and other mosquito repellent products (Tanu and Adholeya 2004). Studies performed with the oil distilled from the leaves of citronella showed high acaricidal activity against the larvae and adult females of the cattle ticks when pure oil and diluted (1:4) in ethanol (Chungsamarnyart and Jiwajinda 1992) was used. Olivo et al. (2008) investigated the effect of the essential oil of citronella on *R.* (*B.*) *microplus* engorged females and reported 90% of egg laying inhibition at the concentrations of 1, 10, 25 and 100%. Other study carried out with *C. winterianus* essential oil demonstrated that the concentrations of 6.1 and 4.1% were lethal to engorged females and larvae of *R.* (*B.*) *microplus*, respectively. Hatching was not observed in the eggs from the engorged females treated with the essential oils at the concentration of 7.14% and egg lying was inhibited by the oil at 10%. Citronellal, geraniol and citronellol were the main components identified in the essential oil (Martins 2006).

Schinus molle L. (Anacardiaceae) is a tree from the South America, originating from Peru (Huerta et al., 2010). It has active substances, such as terpenes, tannins, alkaloids, flavonoids, essential oils, and oleoresins, mainly in the leaves and fruits (Ferrero et al. 2007). Ferrero et al. (2006 2007) studied the insecticidal and repellent effects of the extracts from *S. molle*. Hayouni et al. (2008). Investigated the antimicrobial activity of pepper tree essential oil.

In this work, the essential oils of *C. winterianus* and *S. molle* were fractionated by vacuum distillation (Babu and Kaul 2007) and tested against *R.* (*B.*) *microplus*.

MATERIAL AND METHODS

Plant material and extraction of the essential oil

The aerial parts of *C. winterianus* were collected from the Experimental Farm of Tekton Óleos Essenciais Ltda in the state of Rio Grande do Sul. The essential oil was extracted from the fresh aerial parts by steam distillation in the Tekton industrial distillery (Cassel and Vargas 2006; Cassel et al. 2009). The average yield of the extraction, 0.62% p/p, was calculated on a wet weight basis from the relationship between the essential oil mass and mass of leaves of the fresh aromatic plants.

The *S. molle* essential oil was extracted from the fresh plant (fruit – 25% p/p; leaves – 65% p/p; stems – 10 % p/p) by steam distillation. The average yield of the pepper tree essential oil on industrial scale was 0.30% p/p. The fresh plants were collected and the essential oil was extracted in Experimental Center Angel Gallardo.

Essential oil fractionation

The essential oil was fractionated using a vacuum distillation apparatus (Fig. 1). A volume of 300 mL was introduced in a round bottom flask that presented three outputs: output B had a capillary tube aiming to control and stabilize the ebullition inside the round bottom flask; the output TC1 was used to introduce a temperature sensor for the control of essential oil temperature during the distillation in order to avoid very high temperatures, and the third output was employed to connect the packed column.

The fractionating column was 1.5 m in height and was packed with titanium alloy. The packing material was selected in order to protect against the corrosion. The measure of temperature at the top of the fractionating column was determined by the thermocouple with a precision of ± 1.5 °C. Temperatures in the condensers were controlled by a thermostatic bath (TECNAL, Model TE-2000) and the maintenance of the system pressure was made by vacuum pump (TECNAL, Model TE-058).

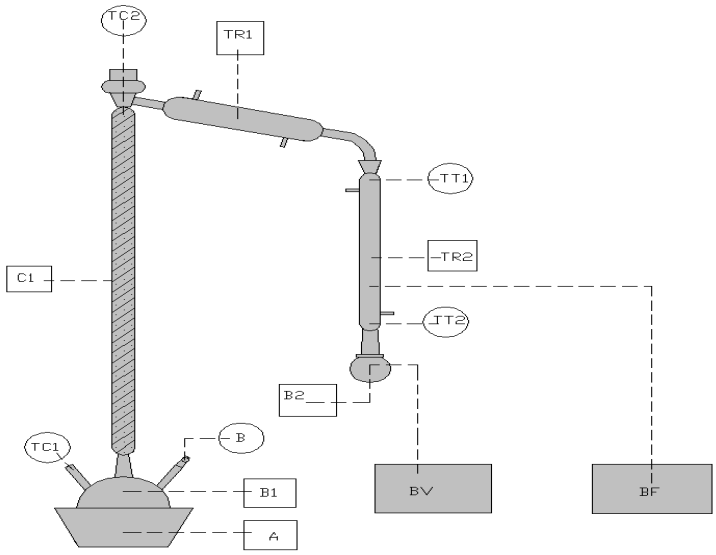

Figure 1- Schematic diagram of the vacuum distillation apparatus: B1 - round bottom flask; C1 – packed column; TR1 and TR2 – condenser, B2 – sample flask, BV – vacuum pump; BF – thermostatic bath, TC1, TC2 – temperature controllers.

Qualitative and quantitative analyses

The quantitative and qualitative analyses of the oils were performed by the capillary GC/MS on an Agilent 7890A mass selective (MS) detector system operating at 70 eV using a HP-5MS capillary column (30 m x 0.25 mm x 0.25 μm). Injector and detector temperatures were set at 60 and 325/350 ºC, respectively; the oven temperature was programmed from 60 - 300 ºC in HP-5 column and 60 - 230 ºC.

Compound identification was based on a comparison of the retention indices (determined relatively to the retention times of a series of *n*-alkanes) and mass spectra with those of authentic samples and/or with literature data (Adams, 2007).

Acaricidal tests

The essential oils and fractions were serially diluted in ethanol (95%) in order to obtain the concentrations of 25.0, 12.5, 6.25, 5.0, 2.5, 1.25 and 0.625 μL/mL. The Mozo strain used as susceptible reference strain was provided by the IPVDF (Instituto de Pesquisas Veterinárias Desidério Finamor). Engorged females *R.* (*B.*) *microplus* were collected from the infested animals, washed with water and dried in paper towel. The average weight of engorging ticks was 0.3 g. These females were incubated at 27–28 ^{o}C and 70–80% relative humidity for two weeks until the egg laying. These eggs provided the larvae used for the larval immersion test - LIT (Ribeiro et al. 2007).

The LIT was conducted by placing approximately 100 embryonated eggs (0.005 g) into pockets (1.0 cm x 1.5 cm) made with TNT fabric. The pockets were incubated at 27–28 ^{o}C and 70–80% relative humidity for 14 days, until the eggs started to hatch. After another 14 days, the pockets containing the larvae ready for testing were immersed for 5 minutes in 10 – 20 mL of the test solutions. Ethanol (95%) was used as control. After 1 h to allow the solvent to evaporate, the pockets were incubated at 27–28 ^{o}C and 70–80% relative humidity for 48 h and then larvae (alive and dead) were counted to assess the percent mortality. Each treatment contained three replicates.

RESULTS AND DISCUSSION

The citronella and pepper tree essential oils have been widely used as insect repellent. Several studies have been done and the efficacy of the oils against mosquitos and ticks has been demonstrated. Nevertheless, no study has been carried out with the essential oil fractionated by vacuum distillation. In order to prepare the fractions, the essential oils obtained from the aerial parts of *C. winterianus* and from the fruits and aerial parts of *S. molle* by steam distillation were submitted to vacuum distillation at constant pressure varying the temperatures. The average yield of each fraction was calculated from the relationship between the fraction volume and essential oils volume. The results are shown in Table 1.

Table 1 - Yield of *C. winterianus* and *S. molle* essential oil fractions obtained by vacuum distillation.

	C. winterianus		***S. molle***	
Fractions	**Temperature Range (ºC)**	**Yield (% v/v)**	**Temperature Range (ºC)**	**Yield (% v/v)**
FR1	25-50	10.0	25-35	12.0
FR2	50-75	16.5	35-50	18.0
FR3	75-100	15.0	> 50	70.0
FR4	100-125	13.5		
FR5	> 125	45.0		

The percentages of each component of the *C. winterianus* and *S. molle* essential oils and fractions obtained by vacuum distillation are reported as raw percentages without standardization in the Table 2 and Table 3, respectively. Some compounds not detected in the essential oils and present in the heavy fractions could be formed by thermal degradation or they were detected in these fractions since the main components, more volatiles, were removed in the former fractions.

Laboratory tests were carried out to determine the toxicity of the citronella and pepper tree essential oil fractions on the larvae of the cattle tick *R.* (*B.*) *microplus* by the larval immersion test. In the experiments performed with the citronella essential oil and fractions, the samples presented activity in relation to the control. According to the lethal concentration values (LC) presented in Table 4, the fractions FR1 and FR2 were less active than the total essential oil. Fraction FR3 presented LC values similar to those obtained for the essential oil while fractions FR4 and FR5 proved to be the most active samples. Although the chemical composition of the fractions FR4 and FR5 were different, the activities did not differ significantly.

In the present study, the essential oil of *C. winterianus* demonstrated activity against the larvae of the cattle tick in concentrations lower than those previously reported (Olivo et al. 2008). The result could be explained by the variability in the components of the essential oil due to environmental factors or by the susceptibility of the strain.

Martins (2006) demonstrated that when tested separately, citronellal and geraniol exhibited higher acaricidal properties compared to citronellol. Nevertheless, in this work, the fractions containing lower amount of citronellal were the most active. The high activity could be enhanced by the presence of higher amount of geraniol. The activity of the fraction FR5 could be attributed to the geraniol and to the presence of sesquiterpenes and diterpenes, which, due to the higher molecular weight, could remain longer in contact with the parasite.

The other essential oil investigated in this work, the pepper tree essential oil, has been previously studied showing antimicrobial and repellent activity (Ferrero et al. 2006, Ferrero et al. 2007; Hayouni et al. 2008, Huerta et al. 2010). In this work, the toxicity of the *S. molle* essential oil and fractions obtained by the vacuum distillation on larvae of the cattle tick *R.* (*B.*) *microplus* was determined, as shown in Table 5. In relation to fractionation of *S. molle* essential oil, it could be seen that the less volatile fraction, FR3, presented acaricidal activity, while for the other fractions and the essential oil this activity against the cattle tick was not observed.

Table 2 - GC–MS of *C. winterianus* essential oil and fractions obtained by vacuum distillation.

Compound	RI[b]	Area (%[a])					
		EO[c]	FR1	FR2	FR3	FR4	FR5
tricyclene	921	tr[d]	tr	-	-	-	-
α-thujene	924	tr	-	-	-	-	-
α-pinene	932	tr	0.140	-	-	-	-
camphene	946	tr	tr	-	-	-	-
sabinene	969	tr	0.158	-	-	-	-
β-pinene	974	tr	0.112	-	-	-	-
myrcene	988	tr	0.266	tr	0.626	1.147	-
α-felandrene	1002	tr	0.171	-	-	-	-
α-terpinene	1014	tr	-	-	-	-	-
o-cymene	1022	tr	-	-	-	-	-
limonene	1024	4.120	26.193	4.978	0.321	0.155	-
1,8-cineole	1026	tr	-	0.366	0.195	-	-
Z-β-ocymene	1032	tr	-	-	0.285	0.487	-
E-β-ocymene	1044	tr	-	-	0.468	0.873	-
bergamal	1051	0.118	0.427		-	-	-
γ-terpinene	1054	tr	tr	-	-	-	-
p-mentha-3,8-diene	1068	tr	-	-	0.116	0.120	-
terpinolene	1086	tr	0.330	tr	-	tr	-
linallol	1095	0.777	1.511	1.871	1.685	1.000	-
cis-rose oxide	1106	tr	0.110	0.121	0.131	tr	-
trans-rose oxide	1122	tr	-	-	tr	-	-
isopulegol	1145	1.371	2.764	5.241	6.959	3.198	0.263
citronellal	1148	37.987	41.292	53.851	38.711	16.76	1.811
iso-isopulegol	1155	tr	0.424	0.366	-	0.404	-
isomenthone	1162	tr	0.180	1.414	2.023	1.030	-
terpinen-4-ol	1174	tr	0.132	-	-	-	-
α-terpineol	1186	tr	0.133	tr	0.140	0.156	-
n-decanal	1201	0.116	4.748	0.119	0.107	tr	-
citronellol	1223	12.030	0.195	6.994	10.052	15.829	12.445
neral	1235	0.412	6.532	0.331	0.406	0.427	0.139
geraniol	1249	16.606	0.264	9.108	11.848	17.389	17.018
geranial	1264	0.501	-	0.416	0.375	0.288	-
citronellyl formate	1271	tr	tr	tr	0.186	0.282	-
bornyl acetate	1287	tr	-	-	-	-	-
geranyl formate	1298	tr	tr	tr	0.191	0.296	-
citronelic acid	1312	0.196	-	-	-	-	-
8-hydroxy-neo-menthol	1328	0.232	2.560	-	-	-	-
α-cubebene	1345	tr	-	-	0.105	tr	-
citronellyl acetate	1350	2.028	1.293	2.069	4.435*	5.768	4.183
eugenol	1356	1.215	1.131	0.225		0.674	0.954
α-copaene	1374	tr	-	-	tr	0.111	-
geranyl acetate	1379	1.325	1.136	1.867	3.612	5.435	5.253
β-elemene	1389	1.850	1.586	2.537	4.574	6.201	4.870
E- cariophyllene	1417	0.118	-	tr	0.140	0.203	0.195
β-copaene	1430	tr	-	-	-	tr	0.320
α-humulene	1452	0.136	-	tr	0.141	0.220	-
trans-cadina-1(16),4-diene	1475	tr	tr	-	-	0.046	-
γ-muurolene	1478	0.260	0.179	0.130	0.254	0.436	0.706
germacrene D	1484	2.322	-		-	-	-
β-selinene	1489	0.146	-	-	-	0.120	0.296
α-selinene	1498	0.278	0.181	-	-	0.121	0.229
α-muurolene	1500	0.674	-	0.289	0.555	0.908	1.686

(cont. table 2.)

(cont.Table 2)

Compound	RI[b]	Area (%[a])					
		EO[c]	FR1	FR2	FR3	FR4	FR5
germacrene A	1508	0.826	-	-	-	-	-
γ-cadinene	1513	0.709	0.247	0.327	0.607	0.951	2.118
δ-cadinene	1522	2.643	0.793	1.142	1.999	3.148	6.788
α-cadinene	1537	0.144	-	-	0.143	0.240	0.647
elemol	1548	3.811	1.170	1.89	3.400	5.619	21.715
germacrene-4-ol	1574	0.800	tr	-	-	-	-
globulol	1590	0.347	tr	-	-	tr	0.564
1-*epi*-cubenol	1627	0.792*	0.135	0.159	0.245	0.395	0.285
Γ-eudesmol	1630		-	0.219	0.397	0.671	2.119
epi-α-muurolol	1640	1.157	0.192	-	-	0.109	3.802
α-muurolol	1644	0.709*	-	tr	0.176	0.308	0.642
β-eudesmol	1649		-	0.314	0.593	0.998	1.928
α-cadinol	1652	1.716	-	-	-	-	6.143
Total identified		**98.473**	**96.686**	**96.349**	**96.201**	**92.531**	**97.118**

[a] Relative proportions of the essential oil constituents were expressed as percentages obtained by peak-area normalization, all relative response factors being taken as one.
[b] RI (retention index), relative to C9-C20 *n*-alkanes on DB-5MS capillary column (Adams, 2007).
[c] *C. winterianus* essential oil.
[d] 0.01< tr <0.10 %.
* Peak overlap

Table 3 - GC–MS of *S. molle* essential oil and fractions obtained by vacuum distillation.

Compound	RI[b]	Area (%[a])			
		EO[c]	FR1	FR2	FR3
tricyclene	921	0.045	0.106	tr	-
α-thujene	924	1.782	3.861	3.312	0.431
α-pinene	932	8.345	18.479	17.114	3.243
camphene	946	0.32	0.577	0.569	0.124
sabinene	969	34.301	53.578	57.149	28.501
β-pinene	974	2.979	6.94	7.828	5.225
myrcene	988	1.985	1.773	1.694	0.964
α-felandrene	1002	0.178	0.126	0.549	0.334
α-terpinene	1014	1.892	1.107	-	-
o-cymene /p-cymene	1022/1020	4.517	3.177	5.025	8.964
limonene	1024	5.987	4.354	4.610	6.493
E-β-ocymene	1044	0.143	-	-	tr
γ-terpinene	1054	3.598	1.385	0.956	1.524
cis-sabinene hydrate	1065	tr	-	-	0.134
terpinolene	1086	1.049	0.297	0.221	0.615
trans-sabinene hydrate	1098	tr	-	-	0.135
cis-p-menth-2-en-1-ol	1118	0.281	-	-	0.364
trans-sabinol	1137	tr	-	-	0.184
terpinen-4-ol	1174	8.214	1.157	0.683	11.091
p-cimen-8-ol	1179	0.274	-	-	0.702
α-terpineol	1186	0.428	-	-	0.570
trans-piperitol	1207	0.175	-	-	0.248
trans-ascaridol glycol	1266	tr	-	-	0.362
p-cymen-7-ol	1289	tr	-	-	0.334
carvacrol	1298	0.333	-	-	0.384
α-copaene	1374	0.295	-	-	0.394
β-elemene	1389	0.198	-	-	0.585
α-gurjunene	1409	0.182	-	-	0.186

(cont. Table 3.)

(cont. Table 3)

Compound	RI[b]	Area (%[a])			
		EO[c]	FR1	FR2	FR3
E- cariophyllene	1417	2.276	0.171	0.121	2.589
aromandrene	1439	tr	-	-	tr
α-humulene	1452	0.328	-	-	0.324
9-*epi*-E-cariofilene	1464	1.812	0.111	-	2.427
γ-muurolene	1478	0.14	-	-	tr
germacrene D	1484	1.348	-	-	0.623
valencene	1496	0.161	-	-	-
bicyclogermacrene	1500	1.215	-	-	0.426
α-muurolene	1500	0.513	-	-	0.686
γ-cadinene	1513	2.227	0.107	-	2.843
δ-cadinene	1522	1.764	-	-	2.169
α-cadinene	1537	0.153	-	-	-
nerolidol	1561	0.133	-	-	0.111
spathulenol	1577	2.222	-	-	1.787
gleenol	1586	0.344	-	-	-
ledol	1602	0.114	-	-	-
1.10-di-*epi*-cubenol	1618	0.595	-	-	0.580
1-*epi*-cubenol	1627	tr	-	-	-
epi-α-cadinol	1638	4.276	-	-	6.631
TOTAL		**97.132%**	**99.662%**	**99.831%**	**93.286%**

[a] Relative proportions of the essential oil constituents were expressed as percentages obtained by peak-area normalization, all relative response factors being taken as one.
[b] RI (retention index), relative to C9-C20 *n*-alkanes on DB-5MS capillary column (Adams. 2007).
[c] *S. molle* essential oil.
[d] 0.01< tr <0.10 %.

Table 4 - Lethal concentration values (μL/mL) of the essential oil of *C. winterianus* and fractions obtained by vacuum distillation on the larvae of *R.* (*B.*) *microplus.*

LC (μL/mL)	FR1	FR2	FR3	FR4	FR5	*C. winterianus* EO
LC_1	2.42	1.75	0.63	ND	ND	0.70
LC_{50}	4.37	4.24	3.49	1.19	1.34	3.30
$LC_{99.9}$	6.86	6.85	6.41	4.38	4.19	5.95

ND: not verified; LC values were determined by linear regression; EO essential oil.

Table 5 - Lethal concentration values (μL/mL) of the essential oil of *S. molle* and fractions obtained by vacuum distillation on the larvae of *R.* (*B.*) *microplus*

LC (μL/mL)	FR1	FR2	FR3	*S. molle* EO
LC_1	ND	ND	5.13	ND
LC_{50}	ND	ND	8.8	ND
$LC_{99.9}$	ND	ND	12.5	ND

ND: not verified; LC values were determined by linear regression, OE essential oil.

Results from this study showed that the less volatile fractions were more active against the tick, which indicated that the contact time of the extract with the larvae was a factor that could influence the toxic effects. It was also observed that the chemical composition of the fractions varied significantly, which indicated the influence of chemical composition on acaricidal activity. These observations were only possible due to processing by vacuum distillation of essential oils, and thus, obtaining new fraction for the testing of volatile compounds against the cattle ticks.

CONCLUSIONS

From the results, it could be concluded that the less volatile essential oil fractions obtained by vacuum distillation were more active against the cattle tick larvae. Especially for the *S. molle* essential oil, it was observed that a change in the composition caused a significant variation in acaricidal activity. The citronella essential oil fractions presented different behavior against the cattle tick, mainly the fractions FR4 and FR5, when compared with the total essential oil. Results showed that the use of vacuum distillation process could be an alternative in the development of new natural products to be used in the tick control, since the changes in the composition of the essential oils promoted a change in their toxicity against the larvae of *R.* (*B.*) *microplus*.

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PAGINA
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