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Research paper

First report of the effect of *Ocotea elegans* essential oil on *Rhipicephalus (Boophilus) microplus*

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ABSTRACT

Rhipicephalus (Boophilus) microplus is responsible for reducing animal welfare, causing a drop in productive performance and transmitting hemoparasites. The main strategy of tick control is application of synthetic acaricides. However, parasite resistance to these compounds is a major concern. Therefore, the acaricidal and repellent *in vitro* effect of the *Ocotea elegans* essential oil on larvae and adult females of *R. (B.) microplus* were evaluated. The larval packet test (LPT), larval repellency test (RT) and adult immersion test (AIT) were performed. The essential oil was analyzed by gas chromatography (GC/FID) and the structure of the oil's major constituent (92.2% sesquirosefuran) was elucidated by nuclear magnetic resonance. In the AIT, efficacy higher than 90% was detected from the concentration 25 mg/mL upward. In both LPTs performed after 48 h, only the 100 mg/mL concentration resulted in mortalities above 70%. On the other hand, the essential oil caused an average of 95.8% repellency from 0.78 to 100 mg/mL. The LC_{50} in the two LPT (48 h) tests were 59.68 and 25.59 mg/mL, respectively. The LC_{50} and LC_{90} in the AIT were 4.96 and 17.37 mg/mL, and in the RT they were 0.04 and 1.24 mg/mL respectively. We conclude that the essential oil of *O. elegans* leaves has a significant acaricidal effect on engorged females and on larval repellency of *R. (B.) microplus* ticks, and can be a promising alternative for the control of this ectoparasite.

1. Introduction

The importance of the tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) is strongly related to the damages caused by its parasitism, since it directly affects animal welfare, resulting in stress, anemia due to blood spoliation, reduction of productive and reproductive performance, and transmits hemoparasites (causing bovine babesiosis and anaplasmosis). This complex of diseases can cause high mortality rate in herds. So, the economic impact is huge in both dairy and beef sectors. In Brazil, losses due to the cattle tick are estimated at US$ 3.24 billion per year from reduced productivity and costs for treatment (Grisi et al., 2014; Raynal et al., 2013; Rodrigues and Leite, 2013).

The application of chemical acaricides has become indispensable for cattle production and is the main method used for tick control (Cruz et al., 2015; Furtado et al., 2013). However, scientific reports of resistance of *R. (B.) microplus* to the chemical acaricides available in the market are alarming (Higa et al., 2016; Klafke et al., 2017; Raynal et al., 2013). In the search for new drugs, substances of plant origin have demonstrated high efficacy *in vitro* and can be associated with commercial products to prolong the efficacy by reducing the selective pressure for development of resistant tick strains (Chagas, 2015; Roel, 2001). Active molecules from plant species have interesting characteristics for parasite control in animal production systems, such as reduced environmental impact, reduced residues in food, lower cost and delayed parasite resistance (Mello-Peixoto et al., 2013; Roel, 2001).

Ocotea elegans Mez belongs to the Lauraceae, a botanical family with a pantropical distribution. Many Lauraceae species are economically important for containing several substances with use in the pharmaceutical, chemical, food and cosmetic industries, as well as in

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construction, carpentry and papermaking (Marques et al., 2004; Rohwer, 1993). The genus *Ocotea* has about 350 species, found throughout tropical and subtropical America, Madagascar, Africa and the Canary Islands (Quinet, 2005). *Ocotea elegans* Mez, popularly known as "canela-sassafrás" in Brazil, occurs in the southeastern and southern Brazilian Atlantic Forest and in Paraguay (Kropf et al., 2015).

Several classes of compounds have been isolated from the genus *Ocotea*, such as terpenoids, alkaloids, neolignans, allyl phenols, coumarins, and sesquiterpenlactones (Hess, 1995). Recent *in vitro* studies have detected acaricidal effect of *Ocotea lancifolia* and *Ocotea diospyrifolia* etanolic extracts on *R. (B.) microplus* engorged females and larvae (Barbosa et al. 2013; Santos et al., 2013). Likewise, Conceição et al. (2017) evaluated hexane, ethyl acetate and ethanol extracts of *Ocotea acidophyla* leaves and all of them demonstrated acaricidal activity against different stages of the cattle tick. Several species of the Lauraceae family occurring in Brazil have not yet been investigated for their possible biological activities (Marques, 2001; Yamaguchi et al., 2012). Based on this and taking into account the activities of the *Ocotea* genus reported in the literature, the present study aimed to evaluate *in vitro* the acaricidal and repellent effect of *O. elegans* essential oil on *R. (B.) microplus* larvae and adult females.

2. Material and methods

2.1. Collection of plant material and extraction of Ocotea elegans essential oil

O. elegans leaves were collected from different specimens in Restinga de Jurubatiba National Park (PNRJ), in the municipality of Carapebus, Rio de Janeiro state, Brazil (22° 18'32"S, and 41° 66'11"W), during the day, in November and December 2014 and January 2015, under authorization of the federal environmental agency IBAMA (permit 13659-2) The identification of the species was carried out by Dr. Marcelo Guerra Santos and a voucher specimen was deposited at the herbarium of the Teacher Training School of Rio de Janeiro State University, under registration number RFFP 16.873. The material was placed in a 5 L round-bottom flask and brought to boil to allow hydrodistillation for 4h in a Clevenger-type apparatus. The oil was collected and stored at 4 °C for further analyses. This procedure was previously described by Tietbohl et al. (2014).

2.2. Chemical analysis of the essential oil

The essential oil was analyzed by a GCMS-QP5000 (Shimadzu) gas chromatograph, equipped with a mass spectrometer using electron ionization (GC/MS). One microliter of essential oil, dissolved in n-hexane (1:100 mg/μL), was injected into a RTX-5 column (30 m × 0.32 mm × 0.25 μm). The GC conditions were as follows: injector temperature, 260 °C; detector temperature, 290 °C; carrier gas (Helium), flow rate 1 mL/min. and split injection with 1:40 split ratio. Oven temperature was initially 60 °C and then raised to 290 °C at a rate of 3 °C /min. The mass spectrometry conditions were voltage of 70 eV and scan rate of 1 scan/s. The retention indices were calculated by interpolating the retention times of a mixture of aliphatic hydrocarbons (C9–C30) analyzed under the same conditions. Identification of the substances was performed by comparing their retention indices and mass spectra with those reported in the literature (Adams, 2007). The mass fragmentation pattern was also checked with the NIST (National Institute of Standards and Technology) mass spectra libraries. Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (GC/FID), under the same conditions as GC/MS analysis, and percentages were obtained by the FID peak-area normalization method.

The identification and structural elucidation of the main essential oil substance was performed by nuclear magnetic resonance (NMR). The spectra of ^{1}H NMR and ^{13}C NMR were obtained in a Varian VNMRS spectrometer with frequencies of 300 and 500 MHz ^{1}H and 125 MHz for ^{13}C, using deuterated solvent and TMS as internal standard. Chemical shifts (δ) were expressed in parts per million (ppm). Spectral editing was performed using SpinWorks 3.1.5.0 and mestReNova 6.0.2-5475 (Mestrelab Research, 2017).

2.3. In vitro tests with the R. (B.) microplus ticks

These tests were performed under SISBIO research authorization no. 37006-4. The engorged females used in this trial were obtained from cattle of the Embrapa Southeast Livestock research unit, free of acaricide treatment for at least 90 days. For the tests with larvae, 14 to 21-day-old larvae from the engorged females collected from the cattle were used. The resistance of the herd's ticks is monitored annually to verify which acaricidal chemical group should be used in the animals kept by Embrapa. According to the resistance test carried out in 2016, these ticks are resistant to pyrethroids, organophosphates and amidines.

2.3.1. Adult immersion test (AIT)

Engorged females were harvested manually from the cattle, sanitized in tap water, dried with absorbent paper and randomly distributed in groups of 10. The groups had a measured and balanced weight and were immerged for 5 min. in 5 mL of *O. elegans* at concentrations of 100, 50, 25, 12.5 and 6.25 mg/mL, in triplicates. The same procedure was performed for the control groups with distilled water (C1) and 75% ethanol (C2). Afterwards, the engorged females were dried, placed in Petri dishes and kept in an incubator (± 27 °C, RH > 80%) for 15 days for oviposition. After this period, the eggs were weighed and placed to adapted syringes, sealed with hydrophilic cotton and again incubated for another 15 days. Afterwards, the hatch estimation was performed by counting the samples in each syringe (Giglioti et al., 2011). The oviposition index, estimated hatch, reproductive efficiency index and oil efficacy were evaluated according to Drummond et al. (1973).

2.3.2. Larval packet test (LPT)

Filter paper sheets (2 cm × 2 cm) were impregnated with 1 mL of *O. elegans* oil at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg/mL. About 100 larvae were deposited on each sheet impregnated with the solution. The sheets were then folded to form packets and were placed in the incubator (± 27 °C and RH > 80%) (FAO, 1971). Readings were performed after 24 h, counting the dead and live larvae using a vacuum pump with an adapted pipette tip. The same concentrations were also evaluated after 48 h of exposure. Three replicates were performed for each concentration, as well as for the control groups, which were composed of: water (C1) and distilled water + 2% tween 80 (C2).

2.3.3. Larval repellency test (RT)

The repellent activity was evaluated 6 h after immersion of wood toothpicks (25 cm long) in *O. elegans* at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg/mL. The first part of the toothpick near the base (15 cm) had no contact with the solution, while the middle part (15–20 cm) and the upper end (20–25 cm) remained immersed in the solutions for 20 min. in Falcon tubes. The toothpicks of the control groups were immersed in distilled water (C1) and distilled water + 2% Tween 80 (C2). After immersion, each toothpick was fixed in the center of a disposable plastic cup (50 mL) containing plaster, with the wet end up. A quantitative filter paper (JP41, black band, 12.5 cm radius, 28 μm pores) was cut in an octagonal form (± 3 cm each side) and inserted at the toothpick base. Approximately 100 larvae were added to each toothpick base (between 2 and 4 cm). They moved on the toothpick or they were dispersed on filter paper. Each treatment and control was performed in triplicate. Subsequently, larvae of each area were quantified with a vacuum pump, estimating the repellency of *O. elegans*.

Table 1
^{1}H and ^{13}C NMR data of the substance sesquirosefuran, in ppm. Analysis by means of PRESAT, COSY, APT, HSQC and HMBC.

Position	δ H	δ C
1	7.20 (d)	139.00
2	6.15 (d)	124.33
3	–	136.48
4	–	150.00
5	3.28 (d)	39.66
6	multiplet	120.10
7	–	131.56
8	–	26.72
9	–	25.25
10	multiplet	112.93
11	–	113.52
12	1.70 (s)	17.80
13	1.96 (s)	25.79
14	1.59 (s)	9.92
15	1.67 (s)	16.24

d = doublets, s = singlets.

2.4. Statistical analysis

The data of the reproductive parameters, mortality, and repellency caused by the *O. elegans* essential oil on the ticks were evaluated by a completely randomized design using the PROC GLM procedure, whose model included the fixed dose effect and the averages compared by the Tukey test ($p < 0.05$). The determination of the lethal concentrations (LCs) of the essential oil in AIT, LPT and RT was performed through probit linear regression, using the normal distribution and the generalized linear model for binary data (logistic regression), with estimates of the parameters of these equations by maximum likelihood. LC_{50} and LC_{90} were then estimated. The data were analyzed by the statistical SAS software (2002/2010).

3. Results

The *O. elegans* leaves' essential oil presented light yellow appearance and had a pleasant odor. Eleven sesquiterpene compounds were identified, with sesquirosefuran being the major constituent, corresponding to 92.2% of the total relative composition. Table 1 shows the results of the ^{1}H and ^{13}C NMR analyses of the chemical compound sesquirosefuran. The ^{1}H NMR spectrum (Fig. 1) presented four signals relating to the methyls, appearing as singlets at δ 1.96, 1.70, 1.67 and 1.59, a signal for the hydrogen attached to carbon 5, which appeared as a doublet at δ 3.28, a multiplet in the region proximal to δ 5.08, and two doublets at δ 6.15 and 7.20, referring to the hydrogens attached to carbons 2 and 1, respectively. The following signals (in δ ppm) were observed in the ^{13}C NMR spectra: 150.00 (C-4), 139.90 (C-1), 136.48 (C-3), 131.56 (C-7), 124.33 (C-2), 120.10 (C-6), 113.52 (C-11), 112.93 (C-10), 39.66 (C-5), 26.72 (C-8), 25.79 (C-13), 25.25 (C-9), 17.80 (C-12), 16.24 (C-15), 9.92 (C-14). These were consistent with the data found in the SciFinder database, Chemical Abstracts Service.

In the AIT, as expected, the control groups had the highest mean egg weight, in function of the higher oviposition by the females. *O. elegans* oil at concentrations of 25, 50 and 100 mg/mL resulted in the lowest mean egg weights (0.32, 0.06 and 0.03 g, respectively), larval hatching (8%, 0% and 0%, respectively), and reproductive efficiency index (2.63%, 0% and 0%, respectively), achieving the highest efficacies (97.18%, 100% and 100%, respectively). From the concentration of 12.5 mg/mL down, the efficacy was lower, but remained higher than 60% (Table 2).

The effect of the *O. elegans* essential oil on *R. (B.) microplus* larvae can be seen in Table 3, in which the mortality rates obtained in the LPT and the repellency percentages obtained in the RT are shown. In general, LPT1 (24 h) caused low mortality rates, presenting some efficacy from the 12.5 mg/mL concentration up. So, no significant differences between treatments and controls were observed. In LPT2 (48 h), larval mortality started at 3.12 mg/mL, with emphasis on the 100 mg/mL concentration, in which 77.40% mortality was detected, resulting in a statistical difference from the others. LPT3 (48 h) was performed to confirm the results obtained in LPT2. The results followed a similar pattern to LPT2, with larval mortality starting at 3.12 mg/mL concentration, with no difference ($p > 0.05$) between concentrations, except for 100 mg/mL.

Regarding RT, high percentage of larval repellency was obtained at the lowest concentration (0.78 mg/mL). At 1.56 mg/mL, despite having lower mean repellency than 0.78 mg/mL (85.23% and 91.19%, respectively), the numbers did not differ statistically from 0.78 and 6.25 mg/mL concentrations. Repellency of 100% was verified at 50 mg/mL and 100 mg/mL (Table 3).

From the results obtained in each test, it was possible to estimate the lethal concentrations of *O. elegans* essential oil on *R. (B.) microplus*. The LC_{50} in the two LPTs (48 h) were 59.68 (41.34–100.51) and 25.59 (14.23–58.57) mg/mL, respectively. The LC_{50} and LC_{90} in the AIT were 4.96 (2.02–7.22) and 17.37 (12.52–33.23) mg/mL, and in the RT they were 0.04 (0.01–0.15) and 1.24 (0.49–2.14) mg/mL, respectively. These data revealed that in both AIT and RT bioassays, good performance of *O. elegans* was detected, confirming its acaricidal and repellent actions.

4. Discussion

The present study confirmed the presence of sesquiterpenes in the essential oil of *O. elegans* leaves. In previous chemical studies, those substances had been indicated as predominant in the genus *Ocotea*. They have been cited as responsible for the biological activities against insects, ticks and other organisms (Camargo et al., 2013; Moraes, 2012; Nogueira et al., 2014a,b). The ^{13}C and ^{1}H NMR analysis of sesquirosefuran revealed correspondence of the results with the literature data (Masahiro et al., 1982). The substance was identified and appears in the Scifinder library under CAS number 39007-93-7 and its signal spectrum had similar results to those described by Tada et al. (1982). Sesquirosefuran has also been detected in other species of the Lauraceae family, such as *Lindera strychnifolia* (Siebold & Zucc.) Fern.-Vill., *Neolitsea aciculate* (Blume) Koidz., *Neolitsea sericea* (Blume) Koidz,. *Neolitsea zeylanica* (Nees & T. Nees) Merr. (Gottlieb, 1972), and *Actinodaphne longifolia* (Blume) Nakai (Hayashi and Komae, 1980). Various interesting properties have been demonstrated for sesquirosefuran. We can highlight its termiticidal properties (Ozaki, 1999) and potential as a biogenetic precursor of substances with important pharmacological properties, such as the formation of litseaverticillols (sesquiterpenes isolated from a Vietnamese shrub), which have anti-HIV activity (Margaros et al., 2006). In chemical studies, involving auto-oxidation by biomimicry in chemical synthesis, the sesquirosefurans perylene, rosefuran and litseaverticillols presented intermediate potential for synthesis (Margaros et al., 2006; Tada et al., 1982).

This is the first study involving the biological effect of *O. elegans* essential oil on *R. (B.) microplus* ticks. At concentrations of 50 and 100 mg/mL it interfered in oviposition in the AIT as well as hatching, resulting in 100% efficacy. Moreover, 63.9% efficacy at 6.2 mg/mL concentration was observed. These results indicate reduction of the reproductive function of engorged females. Therefore, we believe it should be better studied at the histological level. Our results are close to those of Barbosa et al. (2013), in which ethanol extract of *O. lancifolia* (Schott) Mez leaves and bark at 2 mg/mL showed significant activity on the reproductive parameters of *R. (B.) microplus* engorged females (34.5% efficacy). Conceição et al. (2017) also obtained efficacy higher than 90% with the ethanol extract of *O. aciphylla* (Nees & Mart.) Mez leaves at 50 mg/mL in the AIT against *R. (B.) microplus*. The last authors also verified larvicidal activity of *O. aciphylla* and stated that the larval stage was more susceptible than the adult stage, suggesting this may be

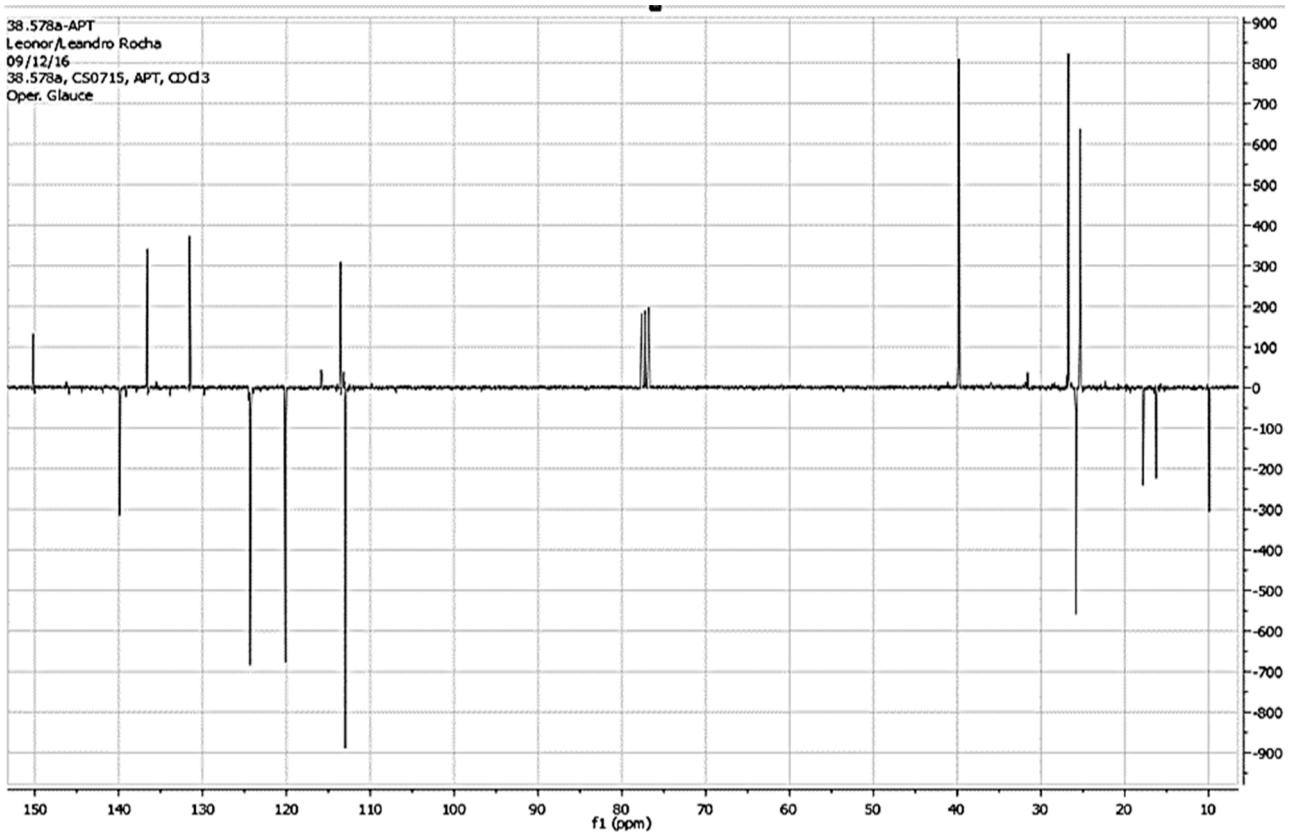

Fig. 1. NMR spectra of sesquirosefuran and its respective ^{13}C carbon chemical shifts (100 MHz, CDCl3).

related to the difference in cuticle composition, which is thinner in larvae (Silva et al., 2009).

In the present study, larval susceptibility was also observed, especially in RT, in which 12.5 mg/mL repelled 99% of the larvae after 6 h. It was also possible to observe high repellency rates at the lowest concentrations. These results are similar to those of Zeringóta et al. (2013), in which eugenol was evaluated (substance present in many species of the Lauraceae family). Repellent rates higher than 90% in the first 7 h were observed for tick larvae at 30, 40 and 50 mg/mL.

The present study did not demonstrate good larvicidal performance of the *O. elegans* essential oil in the LPTs. After 24 h of contact, the mortality rate was only 34.5% at 100 mg/mL, and around 76% at 100 mg/mL in both 48 h tests. On the other hand, studies evaluating ethanol extracts of other species of the *Ocotea* genus have obtained better results on *R. (B.) microplus* larvae. Extracts of *O. diospyrifolia*, for example, produced mortality above 95% in the larval immersion test (LIT) at 40% concentration (Santos et al., 2013). *O. aciphylla* also had efficacy higher than 90% in the LPT at 50 mg/mL (Conceição et al., 2017). These findings may be related to the type of extraction from the plant material, as well as the difference of composition among species, since this can vary widely in the same genus (Chagas, 2015). Therefore, we suggest that other forms of extraction also should be explored for *O. elegans*, to evaluate its potential against *R. (B.) microplus*.

We believe that the sesquiterpene sesquirosefuran, the major constituent of the essential oil, contributed fundamentally to the acaricidal and repellent activities of *O. elegans* against *R. (B.) microplus* in the present study. For this reason, formulations with *O. elegans* oil or with the sesquirosefuran need to be developed and evaluated to find better compounds for cattle tick control in the future. Sesquirosefuran could be incorporated in the synthetic acaricides available, adding repellent action. However, since the yield of the *O. elegans* oil extraction was very low (0.4%), genetic selection to obtain genotypes suitable for commercial production of this species by improving oil production would be interesting. In addition, the search for a route to synthesize sesquirosefuran is also important. Once the sesquiterpene quantity question has been solved, morpho-histological studies of the tick ovaries can confirm the ability to inhibit reproduction, and subsequently formulations based on sesquirosefuran can be evaluated in toxicological and pre clinical trials.

Table 2
Mean of female weights (FW), egg weights (EW), % of larval hatching (LH), reproductive efficiency index (REI) and efficacy of *Ocotea elegans* essential oil on engorged females of *R. (B.) microplus*, evaluated by the adult immersion test (AIT).

Concentration* (mg/mL)	FW (g)	EW (g)	LH (%)	REI	Efficacy (%)
C1 (water)	2.29 ± 0.00a	1.09 ± 0.07a	98.00 ± 0.00a	93.30 ± 6.71a	–
C2 (ethanol)	2.30 ± 0.00a	0.81 ± 0.09a,b	88.33 ± 2.88a	62.19 ± 5.26a,b	–
6.2	2.29 ± 0.00a	0.66 ± 0.14b	55.00 ± 39.05a,b	33.65 ± 25.64b,c	63.93 ± 27.48a
12.5	2.32 ± 0.01a	0.77 ± 0.07b	36.00 ± 15.27b,c	25.13 ± 12.43c,d	73.07 ± 13.32a
25	2.29 ± 0.01a	0.32 ± 0.14c	8.00 ± 10.39c	2.63 ± 3.68c,d	97.18 ± 3.95a
50	2.29 ± 0.02a	0.06 ± 0.11c	0.00 ± 00.00c	0.00 ± 0.00d	100.00 ± 0.00a
100	2.32 ± 0.01a	0.03 ± 0.03c	0.00 ± 0.00c	0.00 ± 0.00d	100.00 ± 0.00a

* Different letters in the column indicate statistically significant difference (Tukey test, $p < 0.05$).

Table 3
Mean efficacy of *O. elegans* essential oil in relation to mortality, verified at intervals of 24 h and 48 by the larval packet test (LPT), and in relation to the repellency, obtained in the larval repellency test (RT).

Concentration (mg/mL)*	Mortality (%)			Repellency (%)
	LPT 1 (24 h)	LPT 2 (48 h)	LPT 3 (48 h)	RT
C1 (water)	0.00 ± 0.00a	0.00 ± 0.00d	0.00 ± 0.00b	19.44 ± 6.89c
C2 (ethanol)	0.00 ± 0.00a	0.00 ± 0.00d	0.00 ± 0.00b	8.07 ± 4.00c
0.78	0.00 ± 0.00a	0.00 ± 0.00d	0.00 ± 0.00b	91.19 ± 7.51a,b
1.56	0.00 ± 0.00a	0.00 ± 0.00d	0.00 ± 0.00b	85.23 ± 3.35b
3.12	0.00 ± 0.00a	10.99 ± 6.37c,d	7.59 ± 5.31b	97.85 ± 2.32a
6.25	0.00 ± 0.00a	5.25 ± 0.70d	37.73 ± 54.13a,b	94.21 ± 5.95a,b
12.5	6.71 ± 9.98a	22.81 ± 4.34b,c	31.12 ± 20.15a,b	99.11 ± 1.52a
25	8.25 ± 4.42a	23.90 ± 7.94b,c	52.26 ± 9.22a,b	99.11 ± 0.86a
50	10.82 ± 17.88a	28.43 ± 8.69b	61.23 ± 33.66a,b	100.00 ± 0.00a
100	34.48 ± 47.61a	77.40 ± 8.40a	76.25 ± 21.90a	100.00 ± 0.00a

* Different letters in the column indicate statistically significant difference (Tukey test, $p < 0.05$).

5. Conclusions

The essential oil of the *O. elegans* leaves presents acaricidal activity, especially on engorged females' reproductive parameters, and has repellent activity against larvae, demonstrating promise for the control of *R. (B.) microplus*. Future studies should also focus on the synthesis of sesquirosefuran, for its inclusion in a formulation with commercially available acaricides, to improve their action.

Statement of animal rights

The experimental protocols that were developed in this study fully complied with the ethical principles of animal experimentation prepared by Ethical Use of Animals of Embrapa Pecuária Sudeste committee (CPPSE/ Protocol No. 02/2014).

Conflict of interest

The authors declare that they have no conflict of interest.

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