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Chemical composition and acaricidal activity of *Ocotea notata* (Lauraceae), an endemic species from Brazil, against the cattle tick *Rhipicephalus microplus*

[Composición química y actividad acaricida de *Ocotea notata* (Lauraceae), una especie endémica de Brasil, contra la garrapata del ganado *Rhipicephalus microplus*]

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Abstract: *Rhipicephalus microplus*, is one parasites that cause severe productivity losses in the cattle industry of Brazil and shows increasing resistance to conventional pesticides. This research aims to study the chemical composition, and acaricidal activity of the essential oil from *Ocotea notata* leaves, a brazilian endemic species, against *R. microplus*. The effect on *R. microplus* engorged adult females was evaluated using the immersion test. The oil reduced the survival by 90% after incubation for 15 days and there was 100% reduction for posture inhibition and reproductive capacity. These results suggest that the *O. notata* essential oil has activity on the *R. microplus*.

Keywords: *Ocotea notata*; *Rhipicephalus microplus*; Parasite control; Essential oil; Acaricide

Resumen: *Rhipicephalus microplus*, es un parásito que causa graves pérdidas de productividad en la industria ganadera de Brasil y muestra una creciente resistencia a los pesticidas convencionales. Esta investigación tiene como objetivo estudiar la composición química y la actividad acaricida del aceite esencial de las hojas de *Ocotea notata*, una especie endémica brasileña, contra *R. microplus*. El efecto sobre las hembras adultas engordadas de *R. microplus* se evaluó mediante la prueba de inmersión. El aceite redujo la supervivencia en 90% después de la incubación durante 15 días y hubo una reducción del 100% para la inhibición de la postura y la capacidad reproductiva. Estos resultados sugieren que el aceite esencial de *O. notata* tiene actividad contra *R. microplus*.

Palabras clave: *Ocotea notata*; *Rhipicephalus microplus*; Control de parásitos; Aceite esencial; Acaricida

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INTRODUCCION

In tropical and subtropical regions worldwide, the livestock industry has substantial economic losses due to the cattle tick, *Rhipicephalus microplus* (Canestrini, 1887) (Acari: Ixodidae). This ectoparasite is responsible for direct losses in the meat and milk production process (Andreotti, 2010; Vinturelle *et al*., 2017). The annual cost of this damage has been estimated at 3.23 billion dollars (Grisi *et al*., 2014). The presence of few natural enemies, easy adaptation to climatic changes and resistance to various synthetic acaricides (Reck *et al*., 2014; Klafke *et al*., 2017) are challenges for controlling this pest. Furthermore, *R. microplu*s is associated with tick fever (Sadness Bovine Parasitic) transmission, a disease caused by the rickettsia species, *Anaplasma marginale* Theiler, 1910, and the protozoans, *Babesia bovis* Babes, 1888 and *Babesia bigemina* Smith; Kilborne, 1893. This disease is endemic from Brazil and there is wide variety of epidemiological factors influencing their occurrence, such as management practices and tick control. Therefore, the study of the tick *R. microplus* is also relevant in veterinary medicine because of the potential transmission to cattle of these three pathogenic agents (Kessler, 2001; Amorim *et al*., 2014).

The first action against this parasite is usually the application of synthetic chemical products such as the organophosphates. Increasing resistance to these acaricides, however, emphasizes the need for new control strategies (Andreotti, 2010; Reginato *et al*., 2017). The use of synthetic pesticides, however, also results in gradual accumulation in the environment and toxicity to humans (Catto *et al*., 2010; Mitra *et al*., 2011: Zhu *et al*., 2014). The search for safer, but effective products has stimulated the study of plant-derived products to control *R. microplus* infestations (Ferreira *et al*., 2018). Furthermore, the increasing emergence of resistance to conventional acaricides, including ivermectin, increases the ability to search for effective new agents (Rodriguez-Vivas *et al*., 2014). The use of essential oils from plants has positive aspects such as his potential low toxicity to mammals, biodegradability and lower pressure of selection of resistance (Koul *et al*., 2008).

Ocotea notata (Nees & Mart.) Mez is a brazilian endemic species popularly known as “white cinnamon” and belonging to the Lauraceae family (Santos *et al*., 2009; Quinet *et al*., 2015). It occurs in the Atlantic Forest, mainly in Restinga vegetation (sandy coastal plains vegetation) (Kropf *et al*., 2015). Several biological activities, such as antibacterial (Garret *et al*., 2007; Costa *et al*., 2015), acaricidal (Conceição *et al*., 2017; Figueiredo *et al*., 2018), antioxidant, acetylcholinesterase (Yamaguchi *et al*., 2012), antifungal and anti-inflammatory (Funasaki & Kato, 2006; Silva *et al*., 2018), have been reported for plants of this genus. The Guarani people, brazilian indigenous, use plants of this genus to treat fever, malaria (Botsaris, 2007), leishmaniasis and similar diseases caused by protozoans (Fournet *et al*., 2007). This genus also has antiplatelet and antithrombotic activity (Ballabeni *et al*., 2007) and acts on the central nervous system (de Sousa *et al*., 2005). The antiherpetic action of the same *O. notata* was also demonstrated (Garrett *et al*., 2012). The present research aimed to determine the chemical composition of the essential oil from the leaves of *O. notata*, and to find safe and effective strategies for its use in controlling the cattle tick by evaluating the *in vitro* effects of this essential oil on engorged *R. microplus* females.

METHODS

Plant Material

The leaves of three specimens of *Ocotea notata* (Nees & Mart.) Mez were obtained from the “Restinga de Jurubatiba” National Park, collected 01/16/2017, on the northwest coast of the state of Rio de Janeiro, Brazil (coordinates 22°14’, 121’’S-41°35’, 807’’W; 22°14’, 107’’S-41°35’, 81’’W and 22°14’, 117’’S-41°35’, 813’’W). The harvesting was carried out under the authorization of SISBIO (Sistema de Autorização e Informação em Biodiversidade, Brazil) n°. 13659-7. The species was identified by Dr. Marcelo Guerra Santos and a voucher specimen (M.G. Santos 2296) was deposited in the herbarium of “Faculdade de Formação de Professores da Universidade do Estado do Rio de Janeiro” (RFFP).

Essential oil extraction

The *O. notata* fresh leaves (815 g) were cut into pieces, mixed and blended with distilled water. The material was placed in a 5 L round-bottom flask for hydrodistillation and subjected to steam distillation for 4 h in a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulphate (Na_2SO_4). The essential oil obtained was stored at 4°C for further analyses (Nogueira *et al*., 2014).

Chemical analysis by GC/MS

The essential oil was analyzed using a gas chromatograph (GC), equipped with a mass spectrometer (MS) by electron ionization (GCMS-QP5000, Shimadzu). The GC conditions were as follows: injector temperature, 260°C; FID temperature, 290°C; carrier gas, Helium; flow rate, 1 mL/minutes and split injection with split ratio 1:40. Oven temperature was initially 60°C and then increased to 290°C at a rate of 3°C/min. One microliter of the essential oil sample, dissolved in hexane (1:100 mg/µL), was injected into a RTX-5 column (I.D: 0.25 mm; length: 30 m; width: 0.25 µm). Mass spectrometry (MS) electron ionization was 70 eV, and the scan rate was 1 scan/s. Retention indices (RI) were calculated by extrapolating the retention times of an aliphatic hydrocarbon mixture (C8-C40), analyzed under the same conditions (Van Den Dool & Kratz, 1963). Substance identification was performed by comparing their retention indices and mass spectra with those reported in the literature (Adams, 2007). The fragmentation pattern of the MS compounds was also compared with the NIST mass spectra libraries. Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (GC/FID), under the same GC/MS analysis conditions (Tietbohl *et al*., 2012).

Acaricidal activity tests

Collection of R. microplus

R. microplus females adult (POA strain) were collected from infested animals from a farm in Rio Grande do Sul (Brazil) without a history of acaricide use. The tick population was maintained by artificial infestations on calves at the Faculdade de Veterinária of the Federal University of Rio Grande do Sul (UFRGS), Brazil. These ticks were collected from a ranch along the border of Brazil and Uruguay in 1992. It has been kept at UFRGS. The Porto Alegre (POA) strain has been widely used as a susceptible reference tick strain since its isolation more than twenty years ago, and has been maintained without exposure to acaricides, as previously described (Reck *et al*., 2009). All experiments were conducted following the guidelines of the Ethics Committee on Animal Experimentation of UFRGS and FEPAGRO. The ticks were thoroughly washed with tap water, dried on filter paper, and used in the adult immersion test.

Tests with engorged females

Tests with engorged females were performed at the Laboratory of Pest and Parasite Studies, in Universidade Federal Fluminense (UFF), Niterói, Río de Janeiro state (Brazil). The essential oil was solubilized at concentrations of 25 mg/mL, 50 mg/mL and 100 mg/mL, in distilled water with 2% dimethylsulfoxide (DMSO). The efficiency assessment was adapted according to Ribeiro *et al*. (2008).

Adult immersion tests (AIT) were undertaken in groups containing fifteen *R. microplus* engorged females of uniform weight. Five replicates were used for testing each concentration of essential oil. The ticks were immersed in each concentration for 1 min, transferred to Petri dishes, and incubated for 15 days in an environmental chamber (Biochemical Oxygen Demand) at 28°C (±1.0ºC) and 80% relative humidity. A negative control group of ticks was treated with 2% DMSO in distilled water (v/v), a non-toxic solvent for ticks (Gonçalves *et al*., 2007). Females mortality was observed. After 15 days, eggs found on the plates were harvested and weighed to calculate oviposition and eclosion inhibition rates (Ribeiro *et al*., 2008; Matos *et al*., 2018).

The results of the test with essential oil were compared with Amitraz and Deltamethrin that were used as acaricide positive controls (250 µg/mL and 25 µg/mL, respectively). The movements of the Malpighian tubules observed under the stereomicroscope were used as one parameter of female survival. The dead ticks were also diagnosed by three specific signs: increasing cuticle darkness, lack of Malpighian tubule movement and hemorrhagic tissue lesions (Drummond *et al*., 1973).

The percentage inhibition of oviposition of each group (15 females) was evaluated after 15 days of treatment as described by Ribeiro *et al*. (2008) and was calculated as follows:

Reproductive Index (RI) = average weight of eggs laid (g)/average weight of females before treatment (g).

Inhibition of Oviposition (IO%) = RI of control group – RI of treated group/ RI control group x 100.

Statistical analysis

Data were expressed as the mean ± standard error of the mean. Groups were compared using "One-way analysis of variance" (ANOVA). When *p*>0.05, differences between treatment and control group were not considered statistically significant. Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, USA). Probit analysis was performed with a 95% confidence interval for all determinations, using SPSS 8.0 for Windows.

Table No. 1

Compound	RI literature	RI calculated	%
trans-muurola-4(14),5-diene	1493	1489	15.61
bicyclogermacrene	1500	1505	12.79
tricyclene	921	918	8.75
(E)-caryophyllene	1417	1426	8.63
β-pinene	974	975	6.52
α-humulene	1452	1459	4.83
δ-cadinene	1522	1529	3.71
limonene	1024	1031	3.51
β-cubebene	1387	1380	3.13
sibirene	1400	1394	2.87
σ-elemene	1335	1341	2.80
14-hydroxy-9-epi-(E)-caryophyllene	1668	1666	2.27
β-elemene	1389	1396	2.20
germacrene B	1559	1563	2.08
myrcene	988	990	1.89
δ-amorfene	1511	1512	1.63
α-cubebene	1348	1353	1.48
guaiol	1600	1598	1.02
α-guaiene	1437	1444	0.91
trans-muurola-3,5-diene	1451	1455	0.91
viridiflorol	1592	1590	0.89
terpinolene	1086	1091	0.79
α-ylangene	1373	1375	0.70
(E)-β-ocimene	1032	1048	0.68
γ-cadinene	1513	1519	0.54
α-terpineol	1186	1193	0.52
cis-muurola-3,5-diene	1449	1448	0.50
α-cadinol	1652	1661	0.44
α-phellandrene	1002	1007	0.41
α-pinene	932	936	0.39
sabinene	969	968	0.37
cis-thujopsene	1429	1439	0.37
1,8-cineole	1026	1033	0.33
Monoterpene hydrocarbons		-	23.31
Oxygenated monoterpenes		-	0.85
Monoterpenes: total		-	24.16
Sesquiterpene hydrocarbons		-	65.69
Oxygenated sesquiterpenes		-	4.62
Sesquiterpenes: total		-	70.31
Total identified		-	94.47

Relative abundance (%) of the constituents in the essential oil of *Ocotea notata* leaves from Restinga de Jurubatiba National Park, Rio de Janeiro state, Brazil. RI = Retention Index

RESULTS

Chemical analysis

The yield of essential oil obtained through steam distillation was 0.13% (w/w). A total of 94.47% of the *Ocotea notata* leaf essential oil components were identified. Essential oil from the *O. notata* leaves was composed of a complex mixture, with a total of 33 compounds identified (Table No. 1). The sesquiterpenes hydrocarbons constitute the largest fraction of the essential oil (65.69%) and the major constituents found in the *O. notata* oil were trans-muurola-4(14),5-diene (15.6%) (1), bicyclogermacrene (12.8%) (2), tricyclene (8.7%) (3), (E)-caryophyllene (8.6%) (4) and β-pinene (6.5%) (5) (Figure No. 1).

Acaricidal activity tests

The *O. notata* essential oil showed acaricidal effects against *R. microplus*. After the first day of testing, 95.0% of females treated with 100 mg/mL essential oil solution (EOS) were killed and this reached 100.0% after 15 days (Table No. 2). In the group treated with the 50 mg/mL EOS, only 70.0% of *R. microplus* females were killed on the first day of testing, but this concentration also reached 100.0% females after 15 days of the experiment. The group treated with the 25 mg/mL EOS had 6.7% of *R. microplus* females killed on the first day of testing and a 90.0% mortality rate at the end of the experiment (Table No. 2). Moreover, the ticks showed a high resistance to the commercial pesticides Amitraz (250 µg/mL) and Deltamethrin (25 µg/mL), showing only 25.3% and 62.7% mortality rates in 15 days, respectively (Figure No. 2).

Table No. 2
Percentage of mortality, reproductive index (RI) and inhibition of oviposition (IO) of *Rhipicephalus microplus* females exposed to different concentrations of *Ocotea notata* essential oil

Oil Concentration (%)	FM (%) ± SD	RI ± SD	IO (%)
100 mg/mL	100 ± 0.0[a]	0.00 ± 0.00[a]	100
50 mg/mL	100 ± 0.0[a]	0.00 ± 0.00[a]	100
25 mg/mL	90.0 ± 7.4[a]	0.00 ± 0.00[a]	100
Amitraz (250 µg/mL)	25.3 ± 8.8	0.00 ± 0.00[a]	100
Deltamethrin (25µg/mL)	62.7 ± 12.1[a]	0.07 ± 0.05[a]	83.44
DMSO 2%	10.0 ± 8.9	0.45 ± 0.03	0

FM (%): percentage of female mortality after 15 days; SD: standard derivation; RI: reproductive index; IO (%): percentage of oviposition inhibition. a - Significant difference in relation to the negative control (2% DMSO), using ANOVA one way to *p* < 0.001

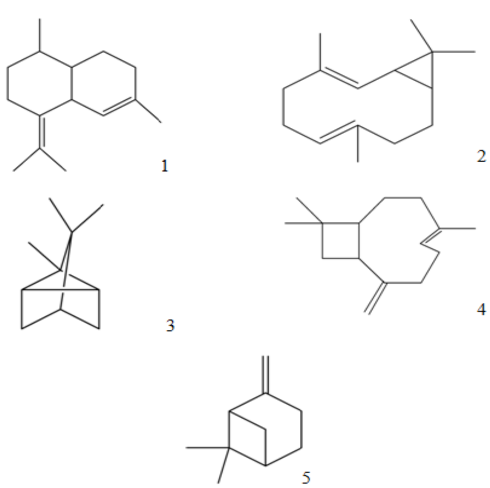

Figure No. 1
The major constituents found in the essential oil of leaves from *O. notata*. 1: trans-muurola-4(14), 5-diene, 2: bicyclogermacrene, 3: tricyclene, 4: (E)-caryophyllene and 5: β-pinene

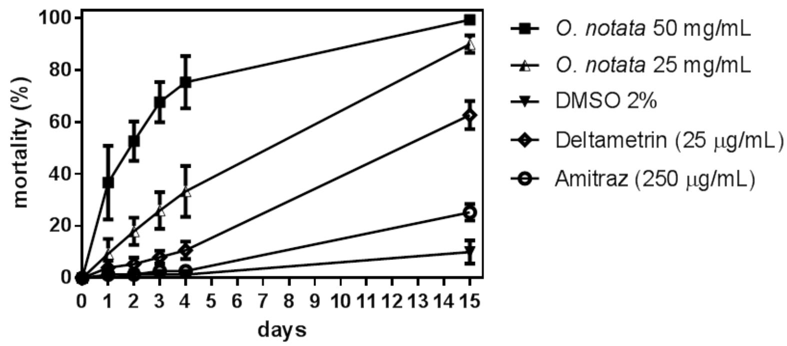

Figure No. 2
Percentage mortality over 15 days of *R. microplus* engorged females exposed to the essential oil of leaves from *O. notata*. Groups were treated with 50 mg/mL and 25 mg/mL concentrations of essential oil. Negative control group contained 2% DMSO distilled water (v/v). Positive controls were the commercial acaricides, deltamethrin (25 µg/mL) and amitraz. (250 µg/mL) Results are means ± SD of five experiments

Index egg laying and eclosion

O. notata leaf essential oil completely inhibited the egg-laying capacity at all concentrations tested (25, 50 and 100 mg/mL). Amitraz also showed 100% of inhibition and Deltamethrin, 83.44% (Table No. 2).

DISCUSSION

The chemical profile has qualitative and quantitative differences from that published by Garrett *et al.* (2013). These authors reported that the sesquiterpenes constituted the largest fraction of the essential oil, although its proportion was lower than that found in this present study (54.7%), and the main substances of the essential oil were germacrene A (22.7%) and β-caryophyllene (22.9%). These authors still reported that the compounds α-pinene, β-pinene and terpinolene (8.7%, 6.9% and 5.5%, respectively) were the main monoterpene constituents of *O. notata* essential oil. This proportion was also different that found in this work, so that the main monoterpens were tricyclene (8.75%), β-pinene (6.52%) and limonene (3.51%). These differences in chemical profile can suggest internal and/or external factors as possible influences, for example, the collection period, place of collection or even possible different chemotypes. In previous studies about essential oils of the genus *Ocotea*, the sesquiterpenes were indicated as responsible constituents for biological activities. Therefore, these substances may contribute, at least, to the acaricidal activity of *O. notata* essential oil (Camargo *et al*., 2013; Nogueira *et al*., 2014; Figueiredo *et al*., 2018). Moreover, the synergic effects, which could occur in this complex mixture of substances, may be relevant to this biological activity and prevent the development of resistance (Bakkali *et al*., 2008; Bassolé & Juliani, 2012).

Some essential oils with these same compounds in their chemical composition have also been reported acaricidal activity. Ruffinengo *et al*. (2006) showed that the essential oil of *Heterothalamus alienus* exhibited an acaricidal effect against the *Varroa destructor* mite, being this oil composed mainly of β-pinene (44.4%) and trans-muurola-4 (14), 5-diene (9.2%) (Ruffinengo *et al*., 2006). The essential oils of *Araucaria columnaris* and *Drimys brasiliensis*, both with bicyclogermacrene in their composition (16% and 11.8%, respectively), showed toxicity against *R. microplus* larvae (Ribeiro *et al*., 2008; Lebouvier *et al*., 2013).

The use of herbal medicines is growing for development of news strategy to control ectoparasites (Ellse & Wall, 2014; Banumathi *et al*., 2017). Extracts, latex and essential oils from plants have been employed as biopesticides to reduce the negative impact of synthetic chemical pesticides, such as the emerging resistance of *R. microplus*, and the increasing accumulation of toxic residues in meat, milk and the environment (Furlong, 1993; de la Fuente *et al*., 2007; Willadsen, 2008; Pinto *et al*., 2011; Rahman *et al*., 2016).

Essential oils are complex mixtures that comprise many individual constituents, among them volatile terpenes and phenolic compounds, and this composition is highly diversified in different plant species (Dhifi *et al*., 2016). Many reports on the biological activities of essential oils in arthropods have been published and these oils have already proven to be ovicidal, larvicidal, growth inhibitory, anti-nutritive and to have repellent activity in arthropods. *O. notata* is a plant that lives in an environment with sandy soil, poor in nutrients, exposed to high winds, which probably results in the production of secondary metabolites necessary to survive these conditions. Several plant species, such as *Azadirachta indica* A. Juss., *Carapa guianensis* Aubl. (both Meliaceae) and *Ocimum basilicum* L. (Lamiaceae), have been shown to have significant action (acaricidal and egg laying inhibition) against *R. microplus* (Farias *et al*., 2007; Santos *et al*., 2012; Adenubi *et al*., 2016). Martins (2006) also showed that citronella-of-java essential oil (*Cymbopogon winterianus* Jowitt ex Bor) (Poaceae) had acaricidal action with 100% mortality of larvae and engorged females, but with a concentration of 10% higher than *O. notate* (Martins, 2006). Barbosa *et al.* (2013) also made *in vitro* studies with *Ocotea lancifolia* (Schott) Mez leaf extract, a brazilian plant from Cerrado and Pantanal, and recorded moderate activity (34.5%) against *R. microplus* at 2 mg/mL (Barbosa *et al*., 2013).

In this work, *R. microplus* female presented only 25.3% mortality to the commercial drug Amitraz but this acaricide completely inhibited the egg laying. The opposite was observed with Deltamethrin, in which the ticks, despite having a lower survival rate, the surviving females performed egg laying frequently. There are also many reports of tick populations resistant to several acaricides in many regions of Brazil and other countries (Gonzáles, 2002; Guerrero *et al*., 2012; Rodríguez-Vivas *et al*., 2014; Singh & Rath, 2014). The *O. notata* essential oil caused 100.0% tick death and inhibited oviposition prior to killing, breaking the pest reproductive cycle. Thus, this *O. notata* essential oil could be an alternative for use in an integrated control program on the outside of parasitized animal as a biopesticide.

The acaricidal activity of *O. notata* essential oil also interferes with the development of the eggs of *R. microplus* and provides additional relevant data regarding its use in complementary programs for pest control.

The results presented here show that the *O. notata* leaf essential oil provides promising new strategies for tick control since the product has been shown to have toxic action on females and on the reproduction of *R. microplus.* However, a series of investigations must be made, such as standardization for species cultivation and development of a stable formulation.

CONCLUSION

This work shows the use of *O. notata* leaf essential oil for acaricidal activity providing data relevant to its possible use in complementary pest-control programs. Biopesticide has the potential to become the future tool to reduce problems such as resistance and toxic residues. The activity of plant materials can be due to impact of a single compound or combination of one or more active components present in the plant. Thus, this study contributes to the knowledge of phytochemicals and their interaction with the biological activities of *O. notata*.

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