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In vitro acaricide activity of Ocotea aciphylla (Nees) Mez. (Lauraceae) extracts and identification of the compounds from the active fractions

Rodrigo Souza Conceição[a], Monique Marylin A. de A. Carneiro[a], Isabella Mary Alves Reis[a], Alexsandro Branco[a], Ivo Jose Curcino Vieira[b], Raimundo Braz-Filho[b,c], Mariana Borges Botura[a,*]

[a] Departamento de Saúde, Universidade Estadual de Feira de Santana, Av. Transnordestina s/n, 44036-900, Feira de Santana, BA, Brazil
[b] LCQUI-CCT, Universidade Estadual do Norte Fluminense, 28013-600, Campos dos Goytacazes, RJ, Brazil
[c] LCQUI-CCT, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes-RJ/DEQUIM-ICE, Universidade Federal Rural do Rio de Janeiro, 23894-374, Seropédica, RJ, Brazil

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ABSTRACT

The *in vitro* acaricide activity of hexane, ethyl acetate and ethanol extracts of *Ocotea aciphylla* leaves was investigated by means of an immersion tests using *Rhipicephalus (Boophilus) microplus* engorged females and larvae. All extracts were shown effective against the different stages of the parasite, and the ethanol extract (50 mg/mL concentration) was the most active (more than 90% efficacy in both assays). The ethanolic extract was fractionated using column chromatography with silica gel as stationary phase to furnish several fractions that were tested against larvae of *R. (B.) microplus*. Three fractions showed high acaricidal activity (50 mg/mL), causing between 84.2% and 100% mortality of the larvae. The anticholinesterase effect of these fractions was determined spectrophotometrically using a microtiter assay. The chemical investigation of the active fractions led to the characterization of terpenoids (cadalene **1** and squalene **2**), a phenylpropanoid (dillapiole **3**) and a phenolic mixture containing five proanthocyanidins (**4-8**) and a flavonoid(vitexin-2"-*O*-rhamnoside **9**). Our findings suggest that the *O. aciphylla* has potent acaricidal activity *in vitro* against *R. (B.) microplus*, and that different secondary metabolites are responsible for this effect.

1. Introduction

Rhipicephalus (Boophilus) microplus, a haematophagous ectoparasite, is considered a major problem for cattle production in tropical and subtropical areas worldwide. This tick causes several deleterious effects, such as blood loss, reduction in weight gain, decreased milk production and direct damage to hides. It also transmits agents of babesiosis (*Babesia bovis* and *Babesia bigemina*) and anaplasmosis (*Anaplasma marginale*) (Silva et al., 2013).

The control of *R. (B.) microplus* has mainly involved the frequent use of synthetic chemical acaricides on infested animals. However, the extensive use of these products has led to development of parasite resistance, environmental pollution and contamination of milk products with residues (Furlani et al., 2015). Currently, the search for alternatives to minimize those problems has been proposed, such as the use of natural products. Plants with insecticidal effects can be a promising alternative, with low – or even no – toxicity to mammals, biodegradable characteristics and less chance of development of resistance (Singh et al., 2016). Secundary metabolites play an important role in the protection of plants against insect attacks. The potential acaricidal activity of plant extracts has been the focus of a large number of *in vitro* studies, which have demonstrated repellency, oviposition inhibition and larval mortality against *R. (B.) microplus* (Valente et al., 2014).

The genus *Ocotea*, the largest member of the Lauraceae family, contains about 350 species and is distributed mainly in tropical and subtropical regions, including Brazil. Several biological activities related to the species of this genus have been reported, such as antibacterial, antifungal (Bruni et al., 2004), anticholinesterase (Yamaguchi et al., 2012), and acaricidal (Barbosa et al., 2013; Santos et al., 2013). An important species of this genus is the *Ocotea aciphylla* (Ness) Mez., whose leaves have been traditionally used as a tonic and stomachic (Marques, 2001). Phytochemical investigations showed the presence of neolignans and phenylpropanoids in the trunk of this plant (Felício et al., 1986), yet few scientific reports have been found on their biolocical activities.

* Corresponding author.
E-mail address: mbbotura@uefs.br (M. Borges Botura).

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The present work aimed to evaluate the *in vitro* acaricide activity of extracts and fractions of *O. aciphylla* leaves against *Rhipicephalus (Boophilus) microplus* using adult and larval immersion tests. In order to elucidate the possible mechanism of the acaricidal action of *O. aciphylla* fractions, we evaluated the *in vitro* anticholinesterase effects. The chemical composition of the active fractions was also investigated.

2. Materials and methods

2.1. Chemicals and reagents

Ethyl acetate, *n*-hexane, ethanol, methanol (analytical grade) and silica gel 60 (70–230 mesh) from VETEC were used. The substances utilized for anticholinesterase test were purchase from Sigma-Aldrich® (St. Louis, MO, USA), except for sodium phosphate dibasic and sodium phosphate monobasic, which were obtained from Anidrol (Diadema, SP, Brazil). Analytical thin-layer chromatography (TLC) was performed on commercial aluminum plates coated with silica gel (0.025 mm) (Merck, Darmstadt, Germany).

2.2. Plant material

Leaves of *O. aciphylla* (Nees) Mez (1.25 kg) were collected in the municipality of Rio de Contas (Bahia) in December, 2013. The identification of the plant species was carried out by a botanist and a voucher specimen (HUPES 205865) was deposited at the herbarium of the State University of Feira de Santana, Brazil.

2.3. Extract preparation

Dried and powdered leaves of *O. aciphylla* (1.0 kg) were subjected to extraction by maceration for 72 h using solvents of increasing polarity: hexane, ethyl acetate and ethanol successively. After filtration, the solvents were evaporated under reduced pressure to obtain the hexane (12.58 g, 1.26%), ethyl acetate (19.18 g, 1.92%) and ethanol (24.52 g, 2.45%) extracts.

2.4. Fractionation of the active extract

Nine grams of the ethanolic extract was subjected to fractionation in column chromatography (CC). The chemical constituents were eluted with solvents in order of increasing polarity (hexane, ethyl acetate and ethanol) resulting in 20 fractions. These fractions were analysed by thin layer chromatography (TLC) and the fractions with similar chromatographic profiles were combined, resulting in 17 fractions named F1 to F17. F1, F2, F3 (Hex/EtOAc: 85%), F4 (Hex/EtOAc: 80%), F5 (Hex/EtOAc: 60%), F6 (Hex/EtOAc: 20%), F7 (EtOAc/EtOH: 80%), F8, F9 (EtOAc/EtOH: 60%), F10 (EtOAc/EtOH: 40%), F11 (EtOAc:/EtOH: 20%), F12, F13, F14 (EtOH: 100%), F15 (EtOH/H_2O: 50%) F16, F17 (H_2O: 100%).

The F2 showed high activity against larvae of *R. (B.) microplus* and was subject to fractionation in a flash chromatographic column and eluted with increasing polarity using the system hexane/ethyl acetate and ethyl acetate/ethanol. This procedure resulted in 9 subfractions that were named G1 to G9. The subfraction G1 furnished compounds **1** and **2** (in a mixture) and G4 furnished compound **3**. F9 furnished a phenolic fraction containing compounds **4-9**, in a mixture, after successive recrystallizations.

2.5. Phytochemical analysis

Compounds **1-3** were analysed by Gas Chromatography coupled to Mass Spectrometry (GC–MS) and Nuclear Magnetic Resonance (NMR). The GC–MS analyses were performed using a Shimadzu gas chromatograph model GC 17A equipped with a DB-5 fused silica capillary column (30 m length x 0.25 mm diameter × 0.25 μm film thickness) and coupled to a QP5050A mass spectrometer. The analytical conditions were: initial column temperature was 80 °C, ranging from 80 °C to 280 °C at 10 °C per minute and remained at 280 °C for 40 min; the temperature of the injector and the interface corresponded to 280 °C. The NMR spectra were acquired on a Bruker apparatus, DPX-500 model. ^1H, ^{13}C analysis, HMBC (multiple-bond correlation spectroscopy Heteronuclear) and HMQC (Heteronuclear Multiple Quantum Coherence) were performed with the unit operating at 500 MHz and DETPQ analysis (distorsionless enhancement by polarization transfer including the detection of quaternary nuclei) to 125 MHz. In all analyses, deuterated chloroform (CDCl3) was used as solvent.

Compounds **4-9** were identified by High-Performance Liquid Chromatography (HPLC) using an HPLC apparatus Merck-Hitachi equipped with a VWR HITACHI L- 2130 pump, a VWR HITACHI L-2300 Diode-array detector, and an auto sampler with a 100-μL loop. The data were acquired and processed using Ezchrom Elite software. The mobile phase system corresponded to 0.1% H_3PO_4 in H_2O-acidified water (A) and methanol (B) and the gradients were: time 0–20 min. (75% A and 25% B); 20–25 min. (100% B); 25–35 (75% A and 25% B). The injection volume was 20 μL and the furnace temperature 30 °C. The wavelength range was 200–800 nm with acquisition at 280 nm. The Bruker mass spectrometer (Daltonic ℊ, Amazon Speed ETD model with ESI sources and ion trap analyzer, low resolution) was used for the HPLC–MS analysis.

2.6. In vitro evaluation of acaricide activity

2.6.1. Collection of ticks

Fully engorged females of *R. (B.) microplus* were collected manually from naturally infected cattle, kept on the farm of the Federal Institute Baiano (IFBaiano) located in the city of Itapetinga, state of Bahia, Brazil. Cattle were free of acaricide treatments 45 days prior tick collection. Recent studies, in the state where the ticks were obtained, showed that these parasites are resistant to organophosphates, pyrethroids and amitraz (Raynal et al., 2013).

The engorged females were kept in petri dishes with sufficient aeration and a group of females was used in the adult immersion tests within 24 h after the collection. Another group was incubated (temperature: 26 ± 2 °C and relative humidity of greater than 80%) for oviposition for 2 weeks. The eggs laid were hatched under similar conditions to provide the larvae used for the larval immersion test.

2.6.2. Adult immersion test

The adult immersion test was conducted according to the methodology described by Drummond et al. (1973). Ticks (480 engorged females) were divided into 48 homogeneous groups of 10 females, based on the degree of engorgement and weight. The test was performed with four repetitions for each concentration and control groups.

The engorged females were immersed for 5 min in 5 mL of the following treatments: hexane, ethyl acetate and ethanol extracts from *O. aciphylla* (12.5, 25 and 50 mg/mL), negative control (ethanol 75%) and positive controls (diazinon and fipronil, diluted as recommended by the manufacturer). Following the immersion, ticks were fixed in petri dishes and incubated (26 ± 2 °C and relative humidity >80%) during 15 days for oviposition. After this period, the eggs were weighed, transferred to glass tubes and incubated under the same conditions for larval hatching, which was read visually using a stereomicroscope after 21 days of incubation. The data obtained were used to determine the percentage of reduction on egg oviposition and larval hatching (Barbosa et al., 2013), as well as to calculate