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Original Article

In vitro activity of the essential oil from *Hesperozygis myrtoides* on *Rhipicephalus (Boophilus) microplus* and *Haemonchus contortus*

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ABSTRACT

Commercial antiparasitics have been the main tool to control parasites, but due to the resistance development, plant extracts have been widely investigated to find new molecules. The present study aimed to investigate the *in vitro* acaricide and anthelmintic activities of the essential oil from the aerial parts of *Hesperozygis myrtoides* (A.St.-Hil. ex Benth.) Epling, Lamiaceae. The essential oil was obtained by hydrodistillation analyzed by GC-FID and GC-MS. Four tests were conducted *in vitro* to screen the antiparasitic action of the essential oil. The evaluation on *Rhipicephalus (Boophilus) microplus* was performed with the adult immersion test at concentrations ranging from 0.391 to 25 mg/ml and the larval packet test from 3.125 to 100 mg/ml. For *Haemonchus contortus* the egg hatch test was performed from 0.012 to 25 mg/ml and the larval development test from 0.003 to 0.4 mg/ml. The LC_{50} and LC_{90} values were calculated by Probit. The main components identified in the essential oil were isomenthone (47.7%), pulegone (21.4%), limonene (7.7%), isomenthyl acetate (6.8%) and neoisomenthol (3.9%). In the larval packet test the LC_{50} and LC_{90} were 13.5 and 21.8 mg/ml, respectively. In egg hatch test, the LC_{50} and LC_{90} were 0.249 and 0.797 mg/ml, respectively, while in the larval development test were 0.072 and 0.167 mg/ml, respectively. This is the first report of the *H. myrtoides* evaluation against those parasites. The anthelmintic results proved its efficacy on *H. contortus* encouraging new research with a focus on their main bioactives.

Introduction

Rhipicephalus (Boophilus) microplus, Ixodidae, known as the cattle tick, is an ectoparasite endemic to the tropical and subtropical regions of the world (Rosado-Aguilar et al., 2010; Lopez-Arias et al., 2014). This tick has become included in the genus *Rhipicephalus* after molecular and morphological studies showing the phylogenetic relationship between *Rhipicephalus* and *Boophilus* (Beati and Keirans, 2001; Murrell and Barker, 2003). This parasite has been a major problem for livestock farmers, by setting a very efficient host-parasite relationship. This fact has caused a huge loss and increasing spending on acaricide chemicals to control the infestation. So that control of this parasite is done at farm level and the acaricide products are frequently administrated at a monthly basis throughout the year (Bianchi et al., 2003). The cattle tick infestations cause many economic losses to the livestock farmers due to blood loss, damage in the animal's skin, reduced weight gain and transmission of pathogens such as *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale*, which cause the disease "Tristeza Parasitária", mainly to the calves (Grisi et al., 2014).

The *Haemonchus* genus belongs to the Trichostrongylidae family, which present several species that parasite ruminants, including *Haemonchus contortus* (Rudolphi 1803) Cobb 1898; *Haemonchus similis* Travassos 1914; *Haemonchus longistipes* Railliet et Henry 1909; and *Haemonchus placei* Place 1893 (Jacquiet et al., 1997). *H. similis* is rarely found in parasitic infections of small ruminants such as sheep and goats but *H. contortus* is prevalent and dominant in terms of intensity of infection (Achi et al., 2003). Therefore, sheep and goats show up highly susceptible, with high establishment rate of infection and large excretion of eggs by females, compared to other ruminant species. Its life cycle includes a free life stage (from egg to the infective larvae L_3) and a parasitic

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stage (from L_3 ingested by the host, to the adult phase) (Jacquiet et al., 1998). Natural populations of *H. contortus* inhabit the United States, Brazil, Argentina, Australia, Europe (Scotland, Ireland and France) and Africa (Congo and Mauritania) (Jacquiet et al., 1997). The pathogenesis caused by *H. contortus* is characterized by a severe anaemia due to blood-sucking habit of the worm, leading to a serious impairment of the animal, severe economic losses and even death (Rodríguez et al., 2015).

The parasite resistance to anthelmintic drugs increasingly affects animals such as cattle, goats and sheep, and is widely spread among several genera and phyla of helminthes (Rose et al., 2015; Paraud et al., 2016). The resistance expression occurs when treatment with the drug allows the survival of resistant parasites, which reproduce and contribute with resistant genes to the new generations. The failure of parasite control provides an increase of individuals able to survive to an antiparasitic dose that would be lethal to the majority of the parasites in a susceptible population of the same species (Geary et al., 1999; Sangster, 1999, 2001). This resistance comes mainly from the intensive use of synthetic antiparasitics with sub-dosages and applications at shorter intervals than necessary (Alves et al., 2012; Lopez-Arias et al., 2014).

Control strategies for parasites that minimize the use of synthetic antiparasitics are of increasing importance, as well as the trend towards non-chemical (ecological, organic, green) farming of livestock (Learmount et al., 2016). Secondary metabolites from plants are the subject of researchers in the screening for products that are less harmful to the environment, therefore more sustainable, more selective and with potent effect on a specific target. *Hesperozygis myrtoides* (A.St.-Hil. ex Benth.) Epling belongs to the Lamiaceae family and is popularly known as "poejo" (pennyroyal). This plant is endemic to southeastern Brazil, growing in the Cerrado and Atlantic Forest biomes, at altitude above 1800 m. It possesses a mint aroma that has been associated with its essential oil rich in monoterpene ketones. The main chemical compounds in the essential oil from the aerial parts of *H. myrtoides* are pulegone (19.8–57.3%), isomenthone (14.3–47.7%), limonene (2.1–22.7%) and isomenthyl acetate (0.3–14.3%) (Martini et al., 2011; Castilho et al., 2016).

The essential oils and terpenoids from many plants rich in monoterpene ketones, such as menthone, isomenthone and pulegone, have been extensively tested against some parasites, showing acaricidal and anthelmintic activity (Facey et al., 2005; Tak et al., 2006; Rosado-Aguilar et al., 2010; Amer et al., 2011; Jeon and Lee, 2011; Kamaraj and Rahuman, 2011; Martinez-Velazquez et al., 2011; Carvalho et al., 2012; Chagas et al., 2012; Molefe et al., 2012; Ferreira et al., 2013; Koc et al., 2013). Therefore, the present study aimed to investigate the *in vitro* acaricide and anthelmintic activities of the essential oil from *H. myrtoides* aerial parts.

Material and methods

Plant origin and extraction

Aerial parts of *H. myrtoides* (A.St.-Hil. ex Benth.) Epling, Lamiaceae, were collected in July 2012 in the "Campo dos poejos" (GPS coordinates: 22° 2 31.83′ S/44° 38 30.20′ W, Aiuruoca, MG, Brazil). Plant identification was performed by Dr. Rosana C. Lopes, and voucher specimens were deposited in the Department of Botany, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil (RFA 39448). Samples of 200 g of fresh aerial parts were subjected to hydrodistillation in a Clevenger-type apparatus for 2 h. The essential oil was separated from the hydrolate by centrifugation and use of manual pipette, and then stored under refrigeration in sealed amber flask. The yield was calculated as ml of oil g^{-1} of fresh weight of the plant material.

Chemical analyzes

Chemical analyses of the *H. myrtoides* essential oil were performed at Embrapa Agroindústria de Alimentos. The identification of the essential oil components was carried out by gas chromatography analyses, performed within 2–3 days after essential oil extraction, using an Agilent 6890N gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and HP-5 (5% phenyl – 95% dimethylpolysiloxane) fused silica capillary column (30 m × 0.32 mm × 0.25 μm). The injector temperature was kept at 250 °C and the over temperature programmed from 60 to 250 °C at 3 °C min^{-1}. Detector (FID) was operated at 280 °C. One microliter of a 1% solution of the oil in dichloromethane was injected in split mode (1:50). The percentages of each component were reported as raw percentages without standardization. GC-MS analyses were performed in an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatograph (Palo Alto, CA), fitted with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm), operating in electronic ionization (EI) mode at 70 eV. Transfer line was maintained at 260 °C, while mass analyzer and ion source temperature were held at 150 °C and 230 °C, respectively. Helium (1 ml min^{-1}) was used as carrier gas. Oven temperature programme, injector temperature and split rate were the same as stated for GC analyses. A standard solution of *n*-alkanes (C_7–C_{26}), injected in the same column and conditions as above, was used to obtain the linear retention indices. Individual volatile components were identified by comparison of their mass spectra (MS) and linear retention indexes (LRI) with those reported in literature (Adams, 2007), as well as to the Wiley Registry of Mass Spectral Data, 6th Edition (1994).

In vitro *assays*

Adult immersion test (AIT)

The *in vitro* tests were carried out in the Animal Health Laboratory of Embrapa Pecuária Sudeste. Engorged females of *R. (B.) microplus* were collected from cattle kept at the experimental farm. According to a resistant test performed in 2016, these ticks are resistant to pyrethroids, organophosphates and amidines. The specimens were selected according to their integrity, motility and maximum engorgement. The ticks were then weighed and separated into groups of ten with homogeneous weights, with three repetitions for each concentration including the control group (immersed in distilled water alone) and blank (2% tween 80).

The engorged females were exposed to the oil in concentrations of 25.0; 12.5; 6.25; 3.13; 1.56; 0.78 and 0.39 mg/ml. Females were immersed in the treatment solutions for 5 min, after which were dried on absorbent paper and placed in sterile Petri dishes and incubated (27 ± 1 °C, RH > 80%) to complete the life cycle. At the end of oviposition, the eggs were weighted and transferred to adapted plastic syringes, identified and sealed with cotton. The eggs were put back into the incubator (Tecnal, TE-391 model) under the same conditions for larval hatching. The data obtained were used to determine the percentage of reduction on egg oviposition and larval hatching, as well as to calculate the reproductive efficiency index (REI) and the efficacy of the essential oil (E), according to Drummond et al. (1973).

Larval packet test (LPT)

The larvae were obtained from engorged females collected from the same source and were incubated as described for the AIT. About 100 larvae, with ages between 14 and 21 days, were placed between two sheets of filter paper (2 × 2 cm) previously moistened with 1 ml of the solutions and then enclosed in packets, made of folded sheets of the same filter paper (Chagas et al., 2002 adapted from FAO, 1971). The concentrations tested were 100.0; 50.0; 25.0; 12.5 and

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