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Research paper

Larvicidal potential of piperovatine in the control of cattle tick

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ABSTRACT

Rhipicephalus (Boophilus) microplus is one of the most important ectoparasites in cattle breeding worldwide, causing direct and indirect losses to animals and producers. Chemical acaricides are utilized in the control of cattle tick and the increase in the development of resistance by ectoparasites makes new alternative necessary. Therefore, research studies have been carried out using bioactive molecules that are quickly degraded and that reduce poisoning to appliers and non-target organisms, environmental contamination and development of resistance. Thus, this study aimed to isolate piperovatine from the roots of *Piper corcovadensis*, a native species to Brazil, and to evaluate the larvicidal activity against *Rhipicephalus* (*Boophilus*) *microplus* by larval packet test and in *ex situ* in an open environment. Piperovatine was isolated by classical column chromatography, and identified by ^{1}H and ^{13}C NMR. The lethal concentration (LC) of piperovatine that killed 50% (LC_{50}) and 99% (LC_{99}) of the larvae was determined by Probit analysis. The results indicated LC_{50} 5.17 and LC_{99} 25.41 μg/mL. LC_{99} was tested in *ex situ* in an open environment, and an efficiency of 96.63% was found, indicating that piperovatine kept the larvicidal action determined in *in vitro* test and in open environment. Therefore, this study shows new perspectives to develop products that can be applied in natural conditions to control this ectoparasite.

1. Introduction

Rhipicephalus (Boophilus) microplus (Canestrini, 1887), one of the most important ectoparasites in cattle breeding worldwide, causes annual economic losses of billions of dollars to milk and beef production all over the world, including in Brazil (Miguita et al., 2015). Brazil is the biggest exporter of bovine beef in the world with 1.4 billion tons and a revenue of US$ 5.5 billion in 2016 and almost 30 million slaughtered bovines. Brazil is also the second country with the largest bovine herd in the world with 281 million heads (ABIEC, 2018; IBGE, 2018), but it is estimated that 80% of them are infected by cattle tick, resulting in annual losses of US$ 3 billion to national cattle breeding and causing 33% more losses than all other plagues together (Mesquita, 2013; Grisi et al., 2014).

Cattle tick is a despoiling toxic agent that can cause an animal's weight loss, anemia, decrease in milk and beef production as well as inoculation of toxins and protozoa such as *Babesia bovis*, *B. bigemina* and rickettsia *Anaplasma marginale* through saliva in hosts, resulting in the clinical state of Bovine Parasitic Sadness by hematophagism (Embrapa, 2009; Chagas et al., 2014; Banumathi et al., 2017). Moreover, cattle tick compromises the development of livestock in a region due to the increase in costs with acaricides, labor force, equipment and facilities to keep the health of bovine herds (Hoycaen and Pimenta, 2013; Chagas et al., 2016)

Organophosphates, formamidines, pyrethroids and avermectines are the chemical products utilized in cattle tick control, but their incorrect utilization results in more frequent applications to control tick, causing the accumulation of these compounds, poisoning of humans

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and animals, and contamination of the food and environment, and also promoting the selection of genetically-resistant ticks to different active principles, reducing the shelf life of these products (Furlong, 2005; Abbas et al., 2014; Chagas et al., 2014; Banumathi et al., 2017).

In the past years, research studies on plants have been carried out to prospect bioactive molecules with acaricide properties because they are rapidly degraded, decrease poisoning of human applicators and non-target organisms, reduce environmental contamination, and slow down the development of resistance to these substances (Campos et al., 2012; Valente et al., 2017; Figueiredo et al., 2018).

Most assays that evaluate the efficiency of natural products against this ectoparasite are only carried out *in vitro*; however, field research under controlled conditions are extremely important for scientific investigations (Jonsson, 2004; Valente et al., 2017). The results of *in vitro* laboratorial tests, in *ex situ* in an open environment or *in vivo* conditions present differences due to temperature and humidity variations, active contact with tick larvae, concentration and stability of the biomolecule and factors associated with the host (Chagas et al., 2012; Araújo et al., 2015; Valente et al., 2017). Chagas et al. (2012) emphasized the necessity to investigate promising plants and compounds in *in vivo* studies, and also evaluate the toxic effect to the host.

Piper corcovadensis (Miq.) C. DC. is a native plant to Brazil which belongs to Piperaceae family, and it is popularly known as *João brandinho* and *falso-jaborandi*. It is geographically distributed from the northeast to the south of Brazil (Facundo et al., 2004). It is popularly utilized to treat rheumatism, the flu and coughing; its roots and stems are used to relieve toothache due to its anesthetic action (Parmar et al., 1997). In *P. corcovadensis* extracts, the following amides were identified: piperovatine, piperlonguminine, corcovadine, isopiperlonguminine and isocorcovadine (Costa and Mors, 1981), and also flavonoids like 3',4',5,5',7-penta methoxyflavone, 3',4',5,7- tetra methoxyflavone and 5-dihydroxy-3',4',5',7-tetra methoxyflavone and caffeic acid (Facundo et al., 2004).

Considering the presence of substances from several secondary metabolite classes of this species, a biological investigation is necessary as a promising source to search new bioactives. Thus, this study aimed to isolate piperovatine from crude extract of *P. corcovadensis* roots (CEPC) by column chromatography and evaluate the acaricide activity against *Rhipicephalus (B.) microplus* by larval packet test and in*ex situ* in an open environment.

2. Material and methods

2.1. Plant material

Piper corcovadensis (Piperaceae) roots were collected in May, 2016 in the Ecological station of Caiuá (52° 49' to 52°53'W and 22° 34'to 22° 37' S), Diamante do Norte, Paraná, Brazil. A voucher specimen was identified by Dr. Mariza Barion Romagnolo and deposited at the Herbarium of Nupélia (HNUP) under the registration number 16706.

2.2. Extract preparation and isolation

The roots were dried in an air circulating oven (Quimis®) at a temperature of 40 °C and then pulverized in a knife mill (Usi Ram). Powdered root of *P. corcovadensis* (50 g) was obtained in a Soxhlet apparatus for 2 h using dichloromethane as solvent extractor. Next, it was concentrated under reduced pressure in a rotary evaporator at 40 °C (IKA® RV 10 Basic) and stored under refrigeration at 4 °C; the yield was 3.8%.

For isolation, CEPC (2 g) was submitted to column chromatography on silica gel 60 (0.063-0.2 mm, Macherey-Nagel) and eluted with chloroform, chloroform: ethyl acetate (80:20, 50:50, 20:80), ethyl acetate and methanol in increasing polarity order; the fractions were monitored by UV (Thermo Scientific™ Evolution 60 UV–vis Spectrophotometer) and ^{1}H NMR. The chloroform: ethyl acetate 80:20 fraction (1.41 g) was submitted to column chromatography on Sephadex LH-20 (25–100 μ, Sigma-Aldrich®) and eluted with methanol. The 7–13 fraction (102.3 mg) was submitted to column chromatography on Sephadex LH-20 and eluted with ethyl acetate. The yield of 7–9 fractions was piperovatine (41.5 mg).

2.3. NMR analysis

^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer (Bruker BioSpin GmbH), operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C, with deuterated Chloroform-d (Deuterochloroform, $CDCl_3$) as solvent and tetramethylsilane (TMS) as internal reference.

2.4. Acaricidal activity against Rhipicephalus (B.) microplus

2.4.1. Tick preparation

Engorged females of *R. (B.) microplus* were collected from naturally infested cattle. All animals had not been submitted to any chemical acaricidal treatment for at least 45 days. The average weight of the ticks was 0.20 g and the female ticks were incubated at 27–28 °C and 70–80% relative humidity (RH) for 2 weeks. The eggs were then collected and placed for hatching under the same conditions. The 15-day-old larvae were used in the larval packet test. For acaricidal activityin *ex situ* in an open environment, 100 mg of eggs were weighed for each test tube, placed for hatching under the same conditions, and then 15-day-old larvae were utilized. For the experiment, tubes that had hatching percentage above 95% were selected (Araújo et al., 2015).

2.4.2. Larval packet test (LPT)

LPT was determined using the method recommended by the World Food and Agriculture Organization (FAO et al., 1971) as described by Leite (1988) with modifications. Groups of 100 larvae were placed in a dry filter-paper packet (2 × 2 cm) for the test. CEPC and piperovatine were diluted in aqueous solution with 2% ethanol and the concentration ranged from 1.00 to 40.00 μg/mL. The positive control was prepared at 1.25 μL/mL of a commercial acaricide containing 150,000 μg/mL of cypermethrin, 250,000 μg/mL of chloropyrifos and 10,000 μg/mL of citronellal, and the two negative controls consisted of distilled water and 2% ethanol aqueous solution. Each packet was moistened with 100 μL of the solution (concentration solution of the samples or controls). Packets were incubated at 27–28 °C and 85–95% relative humidity, in the dark for 24 h. Next, they were opened, and the live and dead number of larvae was counted. All tests were performed in triplicate and larvae mortality was determined as the following: LM = [(dead larvae x 100) / (total larvae)] (Gazim et al., 2011). The lethal concentration of 50 and 99% was calculated, using the mortality data.

2.4.3. Acaricidal activity in ex situ in an open environment

The acaricidal activity in *ex situ* in an open environment was determined using the method described by Araújo et al. (2015) with modifications. The lethal concentrations of 99% of CEPC and piperovatine were tested. Positive and negative control solutions were identical to those used in LPT. On the first day, in each pot containing *Brachiaria decumbens*, the leaves were cut at 40 cm of height and tied; then, the tube containing larvae was placed at the base of the grass plants in each pot. After 24 h, the larval migration to the apex of *B. decumbens* leaves was observed, and 10 mL of the solution of the samples was sprinkled in each pot toward the apex where the larvae were agglomerated, afterthe pots were taken to an isolated area outside the laboratory and placed in a row, 50 cm apart from each other, so that the pots were exposed to variations in temperature and humidity of the open environment, and during the day they were exposed to the sun. On the third day, the leaves were cut and placed in a Petri dish to count the living larvae. The larvae that did not present mobility or did not respond to stimuli were considered dead. All tests were performed in triplicate. The number of living larvae found in the control group was