SHORT COMMUNICATION

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Effect of *Piper rivinoides* extract on the control of *Rhipicephalus (Boophilus) microplus*

Efeito do extrato de Piper rivinoides *no controle de* Rhipicephalus(Boophilus) microplus

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ABSTRACT: *Rhipicephalus* (*Boophilus*) *microplus*, is a major economic problem to the livestock industry, particularly as this ectoparasite is becoming resistant to conventional pesticides. Plant extracts, such as those obtained from species of the Piperaceae family, have been used as a natural alternative to control ectoparasites. This study aimed to evaluate the acaricidal and larvicidal action of *Piper rivinoides* extract. In the adult immersion test (AIT), *P. rivinoides* extract was tested at concentrations of 5-50 mg/mL, while for the LPT the extract was tested at concentrations of 0.09-50 mg/mL, and in the ex situ test in an open environment, the *P. rivinoides* extract was tested at a concentration of 6.12 mg/mL, using as a positive control a solution containing 150 µg/mL of cypermethrin, 250 µg/mL of chlorpyrifos and 10 µg/mL of citronellal, and 2% ethanolic aqueous solution was used as a negative control. Data were analyzed with the Tukey test using one-way analysis of variance (ANOVA) and the lethal concentrations were calculated by Probit analysis. The *P. rivinoides* extract in LPT eliminated 100% of larvae at 50 mg/mL after 24 h at 27–28°C. The extract was also effective at the same concentration, killing 100% of engorged female ticks in AIT. Furthermore, in an open environment under semi-natural conditions ex situ, the *P. rivinoides* extract (6.12 mg/mL) had 73.79% efficiency against cattle tick larvae. These results demonstrate the potential acaricidal activity of *P. rivinoides* extract, presenting as a potential natural alternative to combat ectoparasites.

KEYWORDS: larvicide; ticks; plant extract; *Piperaceae;* bovine parasite.

RESUMO: *Rhipicephalus* (*Boophilus*) *microplus*, é um grande problema econômico para a indústria pecuária, particularmente porque esse ectoparasita está se tornando resistente a pesticidas convencionais. Extratos vegetais, como os obtidos de espécies da família Piperaceae, têm sido usados como uma alternativa natural para controlar ectoparasitas. Este estudo teve como objetivo avaliar a ação acaricida e larvicida do extrato de *Piper rivinoides*. No teste de imersão em adultos (TIA), o extrato de *P. rivinoides* foi testado nas concentrações de 5-50 mg/mL, enquanto para o LPT o extrato foi testado nas concentrações de 0,09-50 mg/mL, e no teste *ex situ* em ambiente aberto, o extrato de *P. rivinoides* foi testado na concentração de 6,12 mg/mL, utilizando como controle positivo uma solução contendo 150 µg/mL de cipermetrina, 250 µg/mL de clorpirifós e 10 µg/mL de citronelal, e solução aquosa etanólica 2% foi utilizada como controle negativo. Os dados foram analisados pelo teste de Tukey usando análise de variância (ANOVA) unidirecional e as concentrações letais (CLs) foram calculadas pela análise de Probit. O extrato de *P. rivinoides* em LPT eliminou 100% das larvas a 50 mg/mL após 24 h a 27–28°C. O extrato também foi eficaz na mesma concentração, matando 100% das teleóginas no teste AIT. Além disso, em ambiente aberto sob condições seminaturais *ex situ*, o extrato de *P. rivinoides* (6,12 mg/mL) teve 73,79% de eficiência contra larvas de carrapatos bovinos. Esses resultados demonstram a potencial atividade acaricida do extrato de *P. rivinoides*, apresentando-se como um potencial alternativa natural para combater ectoparasitas.

PALAVRAS-CHAVE: larvicida; carrapatos; extrato vegetal; *Piperaceae*; parasita bovino.

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INTRODUCTION

Ectoparasitoses cause great damage to livestock worldwide, and ticks of the species *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) (Acari: Ixodidae) are considered one of the major problems for the agricultural sector (Paixão *et al.*, 2021). Negative economic impacts in the order of US$ 30 billion each year have been attributed to this tick (Lew-Tabor; Valle, 2016), comprising both productivity losses and expenses related to animal treatment (Cella *et al.*, 2023; Kumar; Rayulu; Kumar, 2017).

Traditional acaricides, synthetic formulas of pyrethroids and organophosphates, when managed inadequately, result in ineffectiveness in controlling these ectoparasites and resistance (Raynal *et al.*, 2020; Garcia-Ponce *et al.*, 2023). With the emergence of ticks resistant to conventional chemical formulas, the interval between treatments is often shortened and higher dosages are required. However, as these compounds are associated with environmental toxicity, concerns have been raised globally (Rosado-Aguilar *et al.*, 2010).

The cattle production chain has sought innovations for the strategic control of these ticks (Andreotti; Garcia; Koller, 2019). With animal and environmental safety at the forefront, natural products have been considered viable alternatives to replace traditional acaricides and insecticides (Medeiros *et al.*, 2019; Bortolucci *et al.*, 2019). Research into the prospecting of plants with bioactive properties for use in One Health approaches to address this issue has grown, particularly regarding to the number of studies exploring products obtained from the secondary metabolism of plants (Marchesini *et al.*, 2020).

Among the numerous plant species in Brazil with therapeutic potential, those of the *Piper* genus stand out. Species of this genus are aromatic plants widely used in cooking (Salehi *et al.*, 2019), and essential oils (EOs) and plant extracts containing secondary metabolites can be extracted from all plant organs. Secondary metabolites obtained from *Piper* spp. have already demonstrated biological properties that include anti-inflammatory (Silva *et al.*, 2008; Duan *et al.*, 2022), leishmanicidal (Vendrametto *et al.*, 2010), antioxidant (Abrahim; Kanthimathi; Abdul-Aziz, 2012), and trypanocidal activities (Veiga-Santos *et al.*, 2013).

Piper rivinoides, the target species of this studyand popularly known as *pariparoba*, is an invasive plant that is endemic to Brazil and is distributed throughout the national territory (Queiroz; Barros; Guimarães, 2020). Recent studies have shown that the predominant compounds of *P. rivinoides* EO are α-pinene, β-pinene, and limonene, and that the EO has significant cytotoxic effects (Fonseca *et al.*, 2021; Machado *et al.*, 2022), and fungicidal (Leal *et al.*, 2019), bactericidal, and protozoacidal activities (Bernuci *et al.*, 2016). Despite the numerous biological activities associated with this species, to date, the insecticidal action of the EO or extracts on cattle ticks has not been investigated. Thus, this study aimed to evaluate the acaricidal effect of the *P. rivinoides* extract on engorged females and larvae of *R.* (*B.*) *microplus*.

MATERIAL AND METHODS

Plant material

Leaves of *P. rivinoides* were collected in April 2014 in Antonina, Paraná, Brazil (25° 25' 44" S, 48° 42' 43" W). One specimen was authenticated and deposited at the Botanical Museum of Curitiba under registration number 396414.

Preparation of extracts

The preparation of extracts was carried out at the Natural Products and Biotechnology Research Laboratory at the State University of Maringá, Maringá, Paraná State, Brazil. The leaves were dried in an air-circulating oven (Quimis Aparelhos Científicos) at a temperature of 40°C and then pulverized in a knife mill (Usiram Indústria Comércio). The *P. rivinoides* leaf powder (100 g) was submitted to extraction in a Soxhlet apparatus for 6 h using dichloromethane as the extraction solvent, which was then concentrated under reduced pressure in a rotary evaporator at 40°C (IKARV 10 Basic®) to give the *P. rivinoides* crude extract (PrCe). The PrCe was stored under refrigeration at 4°C until use.

Tick preparation

Engorged females of *R.*(*B.*) *microplus* were collected from naturally infested cattle from a private dairy in Pérola, Paraná State, Brazil. The collection was carried out manually during routine milking activities, and the ticks were donated to the Natural Products and Biotechnology Research Laboratory at the State University of Maringá, in Maringá, Paraná State, Brazil. Animals had not been submitted to any chemical acaricidal treatment for at least 45 days prior to tick collection.

Adult immersion test (AIT)

The experiment was carried out at the Natural Products and Biotechnology Research Laboratory at the State University of Maringá, Maringá, Paraná State, Brazil. For each PrCe concentration (5–50 mg/mL), 15 female ticks were used (divided into three groups of five) with homogeneous weights (~0.20 g). Each group was immersed in the extract for 5 min (Medeiros *et al.*, 2019), then the engorged females were placed in Petri dishes and incubated at 27–28°C and 70–80% relative humidity (RH) for 2 weeks. This step was to assess whether the extract was effective in causing the mortality of females (mortality rate %), as well as to determine the effect on egg laying (oviposition). The eggs from each engorged female were weighed and placed in a test tube to hatch under the same temperature and humidity conditions. At this stage, the influence of the PrCe on egg hatchability was determined. The negative control consisted of the 2% ethanolic aqueous solution.

From the data obtained (tick mass, egg mass, and hatching rate), the reproductive efficiency (RE) and product efficiency (PE) were determined and calculated according to equations 1 and 2 (Medeiros *et al.*, 2019).

$$RE = \frac{egg\ mass\ (g) \times hatchrate\ (\%) \times 20{,}000}{Mass\ of\ ticks\ (g)} \quad (1)$$

Where, 20,000 is the estimated number of larvae in 1g of eggs (Medeiros *et al.*, 2019)

$$PE = \frac{RE\ of\ the\ negative\ control\ group - RE\ of\ the\ treated\ group\ x\ 100}{RE\ of\ the\ negative\ control\ group} \quad (2)$$

Larvicidal activity *in vitro* by the larval packet test (LPT)

The experiment was carried out at the Natural Products and Biotechnology Research Laboratory at the State University of Maringá, Maringá, Paraná State, Brazil. The LPT method recommended by the Food and Agriculture Organization (FAO) (Stone; Haydock, 1962) was used with some modifications. For the larval immersion test, larvae aged 14 to 21 days were used, coming from engorged females collected from cattle. Groups of 100 larvae were placed in dry filter paper packets (2×2 cm). The PrCe was diluted in aqueous solution with 2% ethanol (0.09–50 mg/mL). A positive control (PC) was prepared containing 150 µg/mL of cypermethrin, 250 µg/mL of chlorpyrifos, and 10 µg/mL of citronellal. The negative control consisted of the 2% ethanolic aqueous solution.

Each packet was moistened with 100 µL of the PrCe and positive and negative controls. Packets were then incubated at 27–28°C and 85–95% RH, in the dark for 24 h. After this, the packets were opened, and the number of live and dead larvae was counted. All tests were performed in triplicate and larvae mortality was determined as the following Mortality % = (dead larvae × 100) / (total larvae) (Leite *et al.*, 1995). The lethal concentrations for 50% (LC_{50}) and 99% (LC_{99}) of the larvae were calculated.

Acaricidal activity *ex situ* in an open environment

The acaricidal activity of the PrCe in an open environment *ex situ* was determined using the method described by Araújo *et al.* (2015), with modifications. Eggs laid by non-treated engorged females (40 mg) were weighed and added to test tubes to hatch into larvae. For *ex situ* testing,40-cm high *Brachiaria decumbens* plants were placed in pots. The pots were placed in a row, 50 cm apart from each other, so that they were exposed to sunlight and the variations in temperature and humidity of the open environment.The species *B. decumbens* was chosen for the experimental model due to its robustness, adaptation to various soils, and extensive use in similar studies.

The test tubes containing larvae were placed at the base of the grass plants in each pot. After 24 h, the larval migration up the *B. decumbens* plants was observed, and 10 mL of the PrCe (at the LC_{99} concentration) was distributed over the location where the larvae had agglomerated.

On the third day, the plants were cut and placed in Petri dishes to count the living larvae. The larvae without mobility or did not respond to stimuli were considered dead. The PC and NC were the same as those used in the LPT, and all tests were performed in triplicate.

The number of living larvae found in the treated groups was compared to that of the NC. The treatment efficacy was calculated using the following formula: Efficacy (%) = (A-B × 100)/A, according to Bittencourt *et al.*, (2003). Where, A is the average number of larvae in the NC, and B is the average number of larvae in the treated group.

Statistical analysis

The data were submitted to analysis of variance (ANOVA) and differences among the group averages were determined by Tukey's test at a 5% significance level. The LC_{50} and LC_{99} values and their respective 95% confidence intervals (CI) were calculated by probit analysis.

RESULTS AND DISCUSSIONS

Several species of the *Piper* genus have been investigated for their different biologicalactivities: antibacterial, antifungal, anthelmintic, and leishmanicidal (Vendrametto *et al.*, 2010; Bernuci *et al.*, 2016; Tharmalingam *et al.*, 2014; Campelo *et al.*, 2018). Piperlongumine (or piplartine), an amide alkaloid, stands out as one of the isolated compounds most studied among the species of this genus (Rafael *et al.*, 2008), as it has already been recognized for its promising anticancer properties (Bezerra *et al.*, 2015; Piska *et al.*, 2018). Another compound isolated from *Piper* spp. is dilapiol, a phenylpropanoid with genotoxic effects against *Aedes aegypti*, the dengue vector mosquito (Rafael *et al.*, 2008), and a predominant constituent in the EO of *P. gaudichaudianum* (Schindler; Heinzmann, 2017).

In the present study, the PrCe demonstrated consistent larvicidal effects on *R.*(*B.*) *microplus*. At the highest PrCe concentration tested in the LPT, 50 mg/mL, larval mortality reached 100%, showing no significant differences concerning the PC group treated with commercial acaricides. At concentrations of 3.12 and 1.56 mg/mL of PrCe no significant differences were observed between the two treatments, with both treatments resulting in larval mortality greater than 50% (Table 1). Larvicidal activity has previously been associated with other species of the *Piper* genus, *P. aduncum* (Pereira Filho *et al.*, 2024; Rafael *et al.*, 2008) and *P. Marginatum* (Santana *et al.*, 2015), with the EOs of these species demonstrating promising results against *A. aegypti* larvae.

Based on the results of the larval mortality rate presented in Table 1, the LC_{50} and LC99 for the PrCe were determined using probit analysis. The LC that caused the death of 99% of the larvae was 6.12 mg/mL (Table 2). Pereira Filho *et al.* (2024), when studying the larvicidal activity of *P. aduncum* essential oils, found an LC_{75} of 5.05 mg/mL for ticks of the *Amblyomma sculptum* species. This value is proportionally lower than that found in the present study, demonstrating that *Piper rivinoides* has significant biocidal activities. In similar studies, the EO of *Eugenia pyriformis* had an LC_{99} of 24.6 mg/mL against *R.* (*B.*) *microplus* larvae (Medeiros *et al.*, 2019), while Gazim *et al.* (2011) reported an LC_{99} of 11.38 g/mL for the EO of *Tetradenia riparia* against the same tick. When comparing the data from the present study with the literature, it could be stated that the PrCe in this study has a higher larvicidal potential than that of EOs of other species. We observed that PrCe demonstrates significant larvicidal potential, which suggests that it is superior to extracts and OEs from different plant species.

Based on the lethal concentrations of the PrCe presented in Table 2, tests were conducted to determine the product's efficiency (PE) against *R.*(*B.*) *microplus* larvae *ex situ* (Table 3). This test aimed to evaluate the chemical stability of the extract when applied in the field, under uncontrolled conditions. The results indicated a PE of 73.79% at a concentration of 6.12 mg/mL, a level of larvicidal effectiveness considered optimal in semi-natural laboratory conditions (*ex situ*). According to the literature, around 95% of all ticks on a property are in the free-living phase, particularly the larval stage (Veríssimo, 2015), this is an important factor that must be considered when developing control strategies for cattle ticks.

The acaricidal efficacy of the PrCe against adult ticks (engorged females) was determined using the AIT method (Table 4). The mortality rate reached 100% for 50 mg/mL of PrCe, with no significant differences compared to the mortality rate of the PC group. According to the Ministry of Agriculture, Livestock, and Supply (Ministério da Agricultura e Pecuária, MAPA) (Brasil, 1997), for new registrations of acaricides in Brazil, a minimum effectiveness of 95% is required; this percentage was surpassed in the present study for the PrCe at the highest concentration tested (Table 4). Lower concentrations, such as 40 mg/mL and 30 mg/mL of PrCe, also exhibited significantly elevated mortality rates of adult ticks (greater than 65%).

Regarding the effects on oviposition and hatchability, a complete inhibition (100%) was observed in both categories at the highest PrCe concentration tested (50 mg/mL), with no significant difference compared to the results of the PC group. At a 40 mg/mL concentration, inhibition was 81.03% for oviposition and 69.89% for hatchability, showing that the PrCe has bioactive properties with relevant biocidal actions even at low concentrations.According to Andreotti, Garcia and Koller (2019), adults can lay approximately three thousand eggs. Therefore, the ability to inhibit >80% of larval procreation is considered an excellent result, reinforcing the possibility that the PrCe can be an alternative for the strategic fight against ticks.

Table 1. Mean mortality of *Rhipicephalus* (*Boophilus*) *microplus* larvae treated with different concentrations of *Piper rivinoides* crude extract.

Concentration (mg/mL)	Mortality rate (%)
PC	100.0 ± 0.00[a]
50.00	100.0 ± 0.00[a]
25.00	82.50 ± 0.77[b]
12.50	70.89 ± 1.46[c]
6.25	60.12 ± 1.36[d]
3.12	54.04 ± 1.91[e]
1.56	52.50 ± 2.31[e]
0.78	42.36 ± 1.20[f]
0.39	35.52 ± 2.09[g]
0.19	19.09 ± 3.37[h]
0.09	0.00 ± 0.00[i]
NC	0.00 ± 0.00[i]

PC: Positive Control; NC: Negative control; Means followed by the same letters do not statistically differ according to Tukey's test at the significance level of $p < 0.05$.

Table 3. Mean number of live *Rhipicephalus* (*Boophilus*) *microplus* larvae recovered from vessels treated with the *Piper rivinoides* crude extract at 6.12 mg/mL and the product efficiency (%).

Treatment	Mean live larvae	Product efficiency (%)
PC	0.00 ± 0.00 [c]	100.00
PrCe	251.57± 27.79 [b]	73.79
NC	960.33 ± 284.51[a]	0.00

Mean followed by the same letter in the column do not differ among them by the Tukey test ($p \leq 0.05$). PrCe =*Piper rivinoides* Crude extract; PC= Positive Control; NC= Negative control.

Table 2. Lethal concentrations (and confidence intervals) of the *Piper rivinoides* crude extract for 50% and 99% of the *Rhipicephalus* (*Boophilus*) *microplus* larvae.

Tratament	Mean	LC_{50} mg/mL (CI)	LC_{99} mg/mL (CI)	X^2
PrCe	3.81 ± 0.56	1.50 (1.26 -1.81)	6.12 (4.25 - 11.59)	44.90

LC_{50}: 50% lethal concentration; LC_{99}: 99% lethal concentration; CI: Confidence interval; The averages followed by the same letter in the column do not differ among them by the Tukey test ($p \leq 0.05$). PrCe=*Piper rivinoides* Crude extract.

Table 4. Mean (± standard error) mortality and product efficiency against *Rhipicephalus* (*Boophilus*) *microplus* engorged females, as well as egg weight and hatchability following immersion of engorged females in different concentrations of *Piper rivinoides* crude extract.

Treatments (mg/mL)	Teleogin mass (g)	Mortality of Teleogin (%)	Eggs weight (g)	Breeding Index	Inhibition of Oviposition (%)	Hatchability (%)	RE (%)	PE (%)
PC	0.171± 0.054	100.00	0.000 ± 0.000 ^{e}	0.00	100.00	0.00 ^{d}	0.00 ^{e}	100.00
50	0.177± 0.046	100.00	0.000 ± 0.000 ^{e}	0.00	100.00	0.00 ^{d}	0.00 ^{e}	100.00
40	0.174± 0.060	66.67	0.017± 0.011 ^{d}	0.102 ± 0.073	81.03	30.41^{c}	5.94 ^{d,e}	94.06
30	0.169 ± 0.040	73.33	0.024 ± 0.009 ^{d}	0.241 ± 0.114	55.42	72.36 ^{b}	20.55 ^{d}	79.45
20	0.175 ± 0.030	40.00	0.052 ± 0.027 ^{b,c}	0.136 ± 0.053	76.79	76.77 ^{b}	45.62 ^{c}	54.38
10	0.169 ± 0.028	6.67	0.054 ± 0.023 ^{b,c}	0.311 ± 0.1269	42.41	92.47 ^{a}	59.09 ^{c}	40.91
5	0.159 ± 0.023	26.67	0.063 ± 0.013 ^{a,b}	0.3939 ± 0.1239	27.05	96.89 ^{a}	76.78 ^{b}	23.22
NC	0.163 ± 0.042	0.00	0.085 ± 0.028 ^{a}	0.5378 ± 0.1594	0.00	96.92 ^{a}	100.00 ^{a}	0.00

PC = Positive control; NC = Negative control; RE = Reproductive efficiency; PE = Product effectiveness; Mean followed by different letters are statistically different by the Tukey test (p > 0.05).

Overall, the most effective treatments in engorged females were observed at higher concentrations of 50 mg/mL and 40 mg/mL, with PEs of 100% and 94.06%, respectively. This level of effectiveness is in line with the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP), which recommends an effectiveness greater than 95% for a quality commercial acaricide (Holdsworth *et al.*, 2006). The PC group achieved a PE of 100%, as expected, in contrast, the NC group had a null PE, clearly showing the dose-dependent effect of treatments (Table 4).

Chauret *et al.* (1996) have reported for the first time in a species of the Piperaceae three known neolignans (conocarpan, eupomatenoid-5, and eupomatenoid-6) isolated from *P. decurrens* via insecticidal bioassay guided fractionation. Sartorelli *et al.* (2001) related the first phytochemical investigation carried out on the species *P. regnellii* on the chemistry of lignans/neolignans. Among the isolated compounds from the ethyl acetate extracts of the roots of *P. regnellii*, are the neolignans conocarpan, eupomatenoid-3, eupomatenoid-5 and eupomatenoid-6. Several compounds of this class have been isolated from Piperaceae species and in the case of *P. regnellii*, phytochemical studies of its roots showed the accumulation of several phenylpropanoids and benzofuran neolignans including conocarpanas the major compound (Sartorelli *et al.*, 2001). Pessini *et al.* (2003) isolated and identified the neolignans conocarpan, eupomatenoid-3, eupomatenoid-5, and eupomatenoid-6 from the extract of the leaves of *P. regnellii*. In another study the antifungal activity of *P. solmsianum* seems to be related mainly to the presence of compounds 2, 3 (neolignans), and 4 (flavonoid), however, it was verified that another active compound, as yet unidentified, exists in the plant. Investigations about the chemistry of *P. rivinoides* indicate the presence of bioactive benzofuran neolignans eupomatenoid-5, eupomatenoid-6, and conocarpan in its extract (Felisberto; Marques; Moreira, 2021).

Based on our observations and data reported in the literature, the insecticidal activity of *P. rivinoides* seems to be related mainly to the presence of compounds eupomatenoid and conocarpan. The results of this study corroborate the biocidal effects already described for plant species of this genus against other groups of parasites (Jeon *et al.*, 2023).

Although the results are promising, it is crucial to carry out further studies to evaluate the applicability of PrCe in natural conditions, particularly considering that piplartine, one of the main secondary metabolites of the *Piper* genus, has already been identified as a toxic molecule for mammals (Piska *et al.*, 2018) and fish, with embryonic malformations observed in zebrafish (*Danio rerio*) (Chauret *et al.*, 1996). Therefore, it is essential to determine the safety profile concerning non-target organisms.Furthermore, it is necessary to analyze the economic viability of the development of a natural acaricide.

CONCLUSION

The crude plant extract of *P. rivinoides* obtained by Soxhlet extraction showed activities against *R.* (*B.*) *microplus* adult ticks and larvae (*in vitro* and *ex situ*), showing that this plant has bioactive molecules with significant acaricidal action in all phases of the cycle. Although more studies must be carried out to evaluate the applicability in natural conditions and determine the safety profile against non-target organisms, the *P. rivinoides* extract rich in neolignans could be an alternative for the development of a potential natural acaricide, replacing traditional synthetic insecticides.

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