

ORIGINAL ARTICLE

Acaricidal activity of extracts from different structures of *Piper tuberculatum* against larvae and adults of *Rhipicephalus microplus*

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ABSTRACT

The strategies to control the cattle tick, *Rhipicephalus microplus* are based mainly on the use of synthetic pesticides. However, the emergence, establishment, and development of resistance of ticks is rendering the main chemical groups ineffective. Finding new molecules to effectively control infestations by *R. microplus* is necessary to maintain the productivity of cattle herds, particularly of taurine breeds established in equatorial and tropical regions of the world. Ethanol extracts from the leaves, stems, and fruits of *Piper tuberculatum* were evaluated in bioassays at concentrations of 50, 25, 12.50, 6.25, 3.12 and 1.56 mg mL⁻¹. The concentrations lethal to 50% of the individuals (LC₅₀) of tick larvae after 24 hours of exposure were 3.62, 3.99 and 5.30 mg mL⁻¹ for fruit, stem and leaf extracts, respectively. Against the engorged females, the highest efficacy rates were obtained at the concentration of 50 mg mL⁻¹, corresponding to 71.57%, 68.38% and 37.03% of the fruit, leaf and stem extracts, respectively. The main effect of the ethanol extracts was on the egg hatching rate of ticks, with a reduction of 55.63% for the fruit and leaf extracts, and 20.82% for the stem extract. The results show that *P. tuberculatum* is a promising source of molecules for use as active ingredients in pesticide formulations for *R. microplus* control.

KEYWORDS: cattle tick, bioassay, active molecules, Piperaceae

Atividade acaricida de extratos de diferentes estruturas de *Piper tuberculatum* sobre larvas e adultos de *Rhipicephalus microplus*

RESUMO

Estratégias de controle do carrapato dos bovinos, *Rhipicephalus microplus*, fundamentam-se na utilização de pesticidas. Os principais grupos químicos utilizados atualmente mostram-se ineficazes devido ao surgimento de populações resistentes. A pesquisa de novas moléculas com eficiência acaricida é uma necessidade para manutenção da produtividade dos rebanhos bovinos estabelecidos em regiões de clima tropical. Avaliamos a atividade de *Piper tuberculatum* para o controle de fêmeas ingurgitadas e larvas de *R. microplus* através de bioensaios de imersão de adultos e de pacotes de larvas. As concentrações avaliadas foram de 50; 25; 12,50; 6,25; 3,12 e 1,56 mg mL⁻¹ de extrato etanólico de folha, talo e fruto de *P. tuberculatum*. As concentrações letais para 50% dos indivíduos (CL₅₀) após 24 horas de exposição de larvas de *R. microplus* foram 3,62; 3,99 e 5,30 mg mL⁻¹ para os extratos etanólicos de fruto, talo e folha, respectivamente. Para fêmeas ingurgitadas, a maior eficácia resultou da concentração de 50 mg mL⁻¹ de extrato de fruto (71.57%). O principal efeito dos extratos etanólicos de *P. tuberculatum* foi sobre a eclodibilidade, com uma redução de 55.63% para extratos de fruto e folha. *P. tuberculatum* mostra-se promissora como fonte de moléculas candidatas a uso em pesticidas, em formulações destinadas ao controle das infestações de *R. microplus*.

PALAVRAS-CHAVE: carrapato dos bovinos, bioensaios, biomoléculas ativas, Piperaceae

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INTRODUCTION

The global economic losses to cattle breeders caused by pests due to lower milk and meat production, hide damage and treatment costs are enormous. The latest data indicate that in Brazil alone, these losses amount to US\$ 6 billion a year, of which 50% are attributed to the cattle tick, *Rhipicephalus microplus* (Canes.) (Grisi *et al.* 2014).

The primary method used to minimize these losses is the application of synthetic pesticides. However, the incorrect and indiscriminate use of these products has caused the development of cattle tick populations that are resistant to the various commercially available chemical groups (Brito *et al.* 2011). The search for new molecules with acaricidal activity is currently one of the greatest challenges to maintaining the sustainability of cattle breeding in tropical regions.

The promising biocidal activity of various plants to control different pathogenic vectors and agents that impair livestock and human health has been demonstrated by various studies (Klauck *et al.* 2014; Custódio *et al.* 2016; Rodrigues *et al.* 2017; Fatemi *et al.* 2017). The active research interest in the chemical components produced by the metabolism of plants has led to the isolation of various substances that take part in the defense mechanisms of plants against attack by pests and diseases. Among these substances, alkaloids, steroids, terpenes, phenylpropanoids, lignans, flavonoids, and amides stand out as promising bioactive molecules from plants for use in the health sciences (Parmar *et al.* 1997).

Approximately 55 plant species have been evaluated regarding their potential use to control *R. microplus* (Borges *et al.* 2011). Among these, *Piper tuberculatum* Jacq. (Piperaceae), popularly known in Brazil as “pimenta d’arda” or “pimenta longa”, stands out as a promising species that produces bioactive molecules with potential use to control pests and diseases that affect both crops (Scott *et al.* 2002; Castro *et al.* 2008; Trindade *et al.* 2012) and livestock (Chagas *et al.* 2012; Lima *et al.* 2014). Extracts of *P. tuberculatum* also have proven action against protozoa of medical importance such as *Leishmania amazonensis* (Ferreira *et al.* 2010) and *Trypanosoma cruzi* (Regasini *et al.* 2009). It also has molluscicidal effect against *Biomphalaria glabrata*, the intermediate host of *Schistosoma mansoni* (Rapado *et al.* 2011; Rapado *et al.* 2013).

This study involved the testing of ethanol extracts of the leaves, stems, and fruits of *P. tuberculatum* for control of *R. microplus*, to determine what structures of this plant have stronger acaricidal activity and can thus be used as a source to synthesize new active ingredients and a bioactive product for control of cattle tick populations. The evaluations were performed through the bioassays: 1) Adult Immersion Test (TIA) for engorged females; and 2) Larval Packet Test (LPT) for the larval stage.

MATERIAL AND METHODS

Collection of the plant material

Leaves, stems, and fruits of *Piper tuberculatum* were collected in the native forest reserve area of the Federal University of Rondônia (63°56'22"W, 8°50'03"S), in the municipality of Porto Velho, state of Rondônia (southwestern Amazon) in January 2013. Prepared botanical vouchers were sent to the Herbarium of the National Research Institute of the Amazon (INPA) for identification confirmation, and registered under number 211724.

Extract preparation and dilution

Leaves (1.40 kg), stems (1.60 kg) and fruits (0.30 kg) were submitted to extraction with ethanol PA (3 L) for seven days, to obtain extracts from leaves (PTLEt, 87 g), stems (PTSEt, 36 g) and fruits (PTFEt, 46.90 g). For the bioassays, the solutions of the three ethanol extracts of *P. tuberculatum* were diluted in ethanol (larval packet test) and water + Tween 20 at 2% (adult immersion test). The gross extracts were weighed and diluted in the solvents with the aid of ultrasound and a vortex agitator to maximize the solubilization. The final concentrations of 50, 25, 12.50, 6.25, 3.12 and 1.56 mg mL⁻¹ were obtained after dissolving the crude extract. The extracts at a concentration higher than 50 mg mL⁻¹ had high density and viscosity, so they were not evaluated.

Tick preparation and bioassays

Engorged females were collected from naturally infested cattle maintained at the Experimental Field of Porto Velho of Embrapa Rondônia (63°50'58"W, 8°48'10"S). Immediately after collection, the ticks were immersed in a 2% sodium hypochlorite solution, dried on paper towels and selected according to integrity, motility, and degree of engorgement.

The larvae used in the larval packet test (LPT) came from 30 engorged females. These ticks were attached dorsally with two-sided tape in Petri dishes and placed in a BOD chamber at a temperature of 27 +/- 1° C and relative humidity above 80% for egg laying. After 18 days of oviposition, the egg masses were removed and placed in plastic tubes plugged with hydrophilic cotton and kept in the BOD chamber under the same temperature and humidity conditions previously described, until hatching of the larvae. Bioassays were conducted in triplicate for each concentration of the extracts, both in larval and engorged female tests.

Larvae sensitivity on impregnated paper

The bioassays to assess the efficiency of the extract on *R. microplus* larvae were performed according to Stone and Haydock (1962), as modified by Miller *et al.* (2002). The packets were made of filter paper and impregnated with each concentration of the stem, leaf and fruit extracts diluted in alcohol. As controls, packets were impregnated with ethanol

(negative control) and technical grade cypermethrin diluted in acetone at a concentration of 25.60 µg mL⁻¹ (positive control).

Approximately 100 larvae were placed in each packet, which was immediately sealed with paper clips and put in the BOD chamber at a temperature of 27 +/- 1° C and humidity greater than 80%, where they remained for 2 hours. After this interval, the packets were opened, and the living and dead larvae were counted in each replicate to calculate mortality.

Immersion test with engorged females

The acaricidal potential of the stem, leaf and fruit extracts of *P. tuberculatum* on engorged females was analyzed by the adult immersion test (AIT), as described by Drummond *et al.* (1973). Each replicate consisted of a group of ten females, with homogeneous weight, immersed for five minutes in 10 ml of extract at each of the concentrations evaluated. As controls we used technical grade cypermethrin diluted in acetone at a concentration of 25.60 µg mL⁻¹ (positive control) and water + Tween 20 at 2% (negative control). After immersion, the engorged females were dried on a paper towel and mounted dorsally in Petri dishes with two-sided tape. The plates were kept in the BOD chamber at a temperature of 27 +/- 1° C and humidity above 80%.

After the end of oviposition, the egg masses of each group were weighed and allocated in labeled plastic syringes plugged with cotton and incubated in the BOD under the same temperature and humidity conditions as described previously for larval hatching. The number of hatched and not hatched eggs were counted after 16 days to determine the percentage reduction of oviposition (% OR) and hatching (% HR) (Gonzales 2003). Estimated reproduction (ER), and treatment efficacy (E) were calculated according to Drummond *et al.* (1973) as follows:

Statistical analysis

The 50% lethal concentration (LC50) was calculated for larvae using packet test mortality data in regression analysis using the Probit test, performed with the BioStat 2009 Professional 5.8.4 software. Variables from the adult immersion test were analyzed using ANOVA with a factorial design of 6x3 (concentration x extracts) followed by the Scott and Knott's test at 5% significance level using the GENES Program (Cruz, 2016).

RESULTS

The leaf and stem extracts caused 100% mortality of the larvae starting at the concentration of 12.50 mg mL⁻¹, while the fruit extract achieved maximum mortality of 96.20% only at the concentration of 50 mg mL⁻¹ (Figure 1). Although the fruit extract did not produce 100% mortality, at the lowest concentrations it performed better than the leaf and stem extracts. The fruit extract also had the lowest LC₅₀ value, 3.62 ± 2.21 (0.73 - 17.98) mg mL⁻¹ (Table 1). The mortality of the negative control was below 5% and of the positive control above 90% demonstrating the quality of the test.

No significant differences of percentage reduction of oviposition were observed in relation to the concentration among the three extracts. A significant difference was only observed for the average percentage reduction of oviposition caused by the PTLEt at concentrations of 3.12 e 1.56 mg mL⁻¹ when compared to the other concentrations (Table 2).

With respect to the percentage reduction of hatching, the comparison of the averages for the extracts PTLEt, PTSEt and PTFEt revealed a significant difference among the concentrations of 1.56, 6.25 and 12.50 mg mL⁻¹ for the three extracts, where the highest averages for these

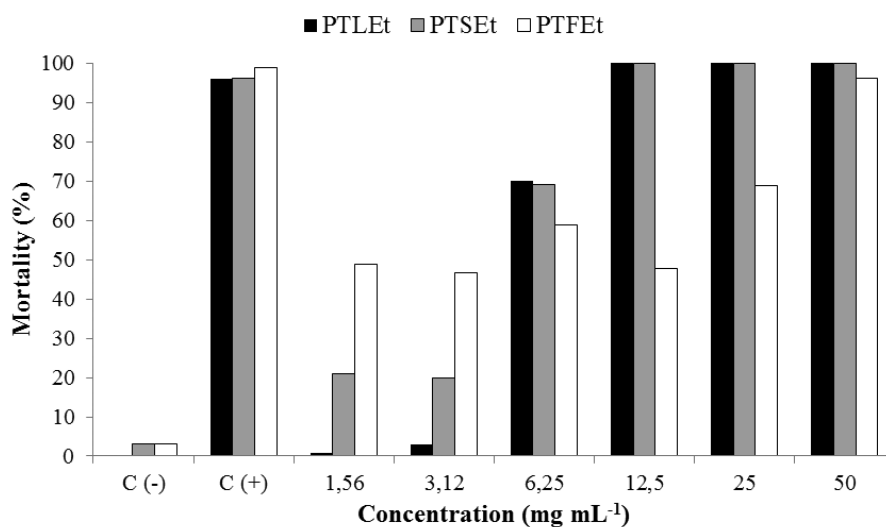


Figure 1. Mortality of *Rhipicephalus microplus* larvae in ethanol extracts of the leaf (PTLEt), stem (PTSEt) and fruit (PTFET) of *Piper tuberculatum* in the larval packet test. Negative control is represented by C (-) and positive control by C (+).

concentrations were observed for PTFEt. For all the extracts, the highest percentages reduction of hatching occurred at the concentration of 50 mg mL⁻¹. For PTFEt, the concentrations of 6.25 and 12.50 mg mL⁻¹ caused the same reduction (the greatest hatching reduction for this extract) (Table 2).

Table 1. LC₅₀ (50% lethal concentration) of the ethanol extracts of the leaf (PTLEt), stem (PTSEt) and fruit (PTFEt) of *Piper tuberculatum* on larvae of *Rhipicephalus microplus*. Values are means of three replicates followed by the standard deviation and the range.

Extract	LC ₅₀ (mg mL ⁻¹)
PTLEt	5.30 ± 0.84 (3.42 - 8.21)
PTSEt	3.99 ± 0.80 (2.18 - 6.60)
PTFEt	3.62 ± 2.21 (0.73 - 17.98)

Table 2. Average percentage reduction oviposition (%OR), hatching reduction percentages (%HR), estimated reproduction (ER), and efficacy (E) in the adult immersion test (AIT) with ethanol extracts of leaf (PTLEt), stem (PTSEt) and fruit (PTFEt) of *Piper tuberculatum* on engorged *Rhipicephalus microplus* females. Concentration (Conc.) in mg mL⁻¹. Numbers are means followed by the standard deviation. Equal upper-case letters in the row and lower-case letters in the column indicate no difference by the Scott-Knott test at 5% significance level.

	Conc.	PTLEt	PTSEt	PTFEt
%OR	50	35.01±16.20 ^{Aa}	26.04±4.98 ^{Aa}	31.91±7.03 ^{Aa}
	25	30.38±3.71 ^{Aa}	27.33±7.09 ^{Aa}	14.72±7.71 ^{Aa}
	12.5	32.05±3.71 ^{Aa}	27.89±11.40 ^{Aa}	29.49±6.03 ^{Aa}
	6.25	27.53±9.36 ^{Aa}	20.56±8.09 ^{Aa}	23.87±2.79 ^{Aa}
	3.12	14.36±3.35 ^{Ab}	26.27±8.62 ^{Aa}	20.92±12.44 ^{Aa}
	1.56	13.27±9.30 ^{Ab}	11.38±6.33 ^{Aa}	20.50±12.25 ^{Aa}
%HR	50	55.63±12.88 ^{Aa}	20.82±5.15 ^{Ba}	55.63±4.26 ^{Aa}
	25	44.37±6.17 ^{Ab}	14.68±4.26 ^{Ba}	38.57±7.38 ^{Ab}
	12.5	1.02±0.59 ^{Cd}	15.02±4.46 ^{Ba}	55.97±4.69 ^{Aa}
	6.25	1.02±0.59 ^{Cd}	20.82±7.26 ^{Ba}	56.97±4.69 ^{Aa}
	3.12	21.50±2.96 ^{Bc}	21.16±2.71 ^{Ba}	51.54±9.23 ^{Aa}
	1.56	18.77±5.25 ^{Bc}	3.75±2.71 ^{Cb}	54.61±8.59 ^{Aa}
ER	50	16.03±8.00 ^{Bb}	31.92±3.72 ^{Ab}	14.41±2.44 ^{Bb}
	25	20.29±2.93 ^{Bb}	32.97±2.63 ^{Ab}	24.09±4.14 ^{Ba}
	12.5	35.26±0.76 ^{Aa}	33.16±3.46 ^{Ab}	14.45±2.41 ^{Bb}
	6.25	37.85±4.80 ^{Aa}	31.61±3.93 ^{Bb}	15.22±1.65 ^{Cb}
	3.12	33.85±3.61 ^{Aa}	32.20±3.12 ^{Ab}	17.55±5.6 ^{Bb}
	1.56	34.62±3.69 ^{Ba}	45.08±2.21 ^{Aa}	16.40±0.64 ^{Cb}
E	50	68.38±15.78 ^{Aa}	37.03±7.34 ^{Ba}	71.57±4.80 ^{Aa}
	25	59.98±5.78 ^{Aa}	34.96±5.20 ^{Ba}	52.48±8.16 ^{Ab}
	12.5	30.45±1.51 ^{Bb}	34.60±6.82 ^{Ba}	71.49±4.75 ^{Aa}
	6.25	25.33±9.48 ^{Cb}	37.65±7.75 ^{Ba}	69.97±3.25 ^{Aa}
	3.12	33.24±7.12 ^{Bb}	36.48±6.14 ^{Ba}	65.38±10.96 ^{Aa}
	1.56	31.71±7.28 ^{Bb}	11.07±4.36 ^{Bb}	67.64±1.26 ^{Aa}

The average estimated reproduction measures declined significantly for the engorged females immersed in PTLEt and PTSEt. For all the extracts, the concentration of 50 mg mL⁻¹ was most effective in reducing the estimated reproduction (Table 2). The concentration of 50 mg mL⁻¹ of both the PTFEt and PTLEt extracts presented the best performance in the AIT, with treatment efficacy levels of 71.57% and 68.38%, respectively (Table 2). There were no viable postures in the female groups of the positive control, demonstrating high pyrethroid efficacy in the evaluated population of *R. microplus*.

DISCUSSION

Leaf extracts of *P. tuberculatum* have previously been studied as insecticides (Trindade *et al.* 2012; Castro *et al.* 2008), however, very few studies have been conducted to assess the use of this plant's extracts against ticks (Chagas *et al.* 2012). The efficiency of an acaricide can vary according to the extraction method, solvent polarity and plant part used to obtain the extract (Lima *et al.* 2014).

All three extracts showed promise against the larvae of *Rhipicephalus microplus*. Chagas *et al.* (2012) observed similar results (LC₅₀=0.41%≈4.10 mg mL⁻¹) using leaf extract of *P. tuberculatum* to control *R. microplus* larvae. Lima *et al.* (2014) obtained a LC₅₀ value approximately 50 times lower than that observed in this study for the ethanol extracts from the fruit of *P. tuberculatum*, and found that extracts obtained using solvents of lower polarity were more effective. Unlike Lima *et al.* (2014), we found that the strongest acaricidal activity of *P. tuberculatum* was produced by the leaf extracts, which can be due to the extraction method or solvent polarity (Silva *et al.* 2009).

The extract from the fruits of *P. tuberculatum* was the most effective in reducing the egg hatching rate in this study, yet Chagas *et al.* (2012) reported a 91.66% egg reduction for a 10% (≈100 mg mL⁻¹) concentration of *P. tuberculatum* leaf extract, while at 5% (≈50 mg mL⁻¹) the efficacy was only 58.61%, close to that observed in our study. The efficacy of fruit extracts of *P. tuberculatum* on *R. microplus* females was of 11.40% using ethanol as solvent, but was 100% and 96.20% using hexane and ethyl ether, respectively, all at a concentration of 75 mg mL⁻¹ in the adult immersion test (Lima *et al.* 2014). The latter authors measured an LC₅₀ of 2.73 mg mL⁻¹ for the ethanol extract of *P. tuberculatum* fruits on *R. microplus* larvae, close to that observed in this study.

Santos *et al.* (2015) assessed the *in vitro* effect of the aqueous extract, hydroalcoholic extract, concentrated hydroalcoholic extract and essential oil of lemongrass (*Cymbopogon wynterianus*) on *R. microplus* larvae and engorged females. They found that the samples had acaricidal action, mainly the essential oil, because starting at a concentration of 12.50% it caused 100% larval mortality, and at 25% it had

an efficacy index of 100% in the test with engorged females, a better result than observed in our study.

Other *Piper* species have been evaluated for control of *R. microplus* larvae and adults. *In vitro* tests showed the toxic potential of the hexane extract and essential oil of the leaves of *P. aduncum* against *R. microplus* larvae and adults (Silva *et al.* 2009). In an evaluation of essential oils of *P. amalago*, *P. mikanianum* and *P. xylosteoides*, the highest mortality of *R. microplus* larvae was observed for *P. mikanianum* (LC₅₀=2.33 µL mL⁻¹), followed by *P. xylosteoides*, while *P. amalago* showed no acaricidal activity on larvae (Ferraz *et al.* (2010).

A wider range of plants should be evaluated regarding their acaricidal potential against *R. microplus* larvae and engorged females. One phytochemical study indicated that 94.84% of the composition of the essential oil of *P. aduncum* consists of dillapiole (Silva *et al.* 2009). Another study found that the majority component of *P. mikanianum* essential oil is apiole (67.88%) and of *P. xylosteoides* is safrol (47.83%), while the essential oil of *P. amalago* contains 20.52% limonene (Ferraz *et al.* 2010). The differences in the chemical constituents isolated from different *Piper* species can be related to the genetic diversity of these species, as well as the foliar age of the plant, edaphoclimatic variations and different extraction methods applied (Facundo *et al.* 2008).

The *P. tuberculatum* extracts demonstrated acaricidal action against larvae and engorged females of *R. microplus*. However, further research is needed to obtain and identify the constituents of these extracts responsible for that action, since biomolecules appear to have great promise for use as biocides against pests and diseases that afflict plants and animals, including humans. Phytochemical studies aimed at isolating these substances, as well as *in vitro* tests with fractions of plant extracts and substances isolated from the metabolism of plants, should be conducted to identify new acaricidal molecules for use in the pharmaceutical industry.

CONCLUSIONS

The ethanol extracts obtained from the leaves, stems and fruits of *P. tuberculatum* presented acaricidal activity *in vitro* against larvae and engorged females of *Rhipicephalus microplus*. The fruit extract presented the best result as acaricide for larvae, followed by the extracts of stems and leaves. Against engorged females, fruit and leaf extracts presented the highest efficacy. Despite high activity against larvae, the stem extract presented low efficacy against engorged females. In general, the fruit of *P. tuberculatum* was the most promising material for the development of acaricides.

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