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In vitro effects of *Pilocarpus microphyllus* extracts and pilocarpine hydrochloride on *Rhipicephalus* (*Boophilus*) *microplus*

Efeitos *in vitro* do extrato de *Pilocarpus microphyllus* e do cloridrato de pilocarpina sobre *Rhipicephalus* (*Boophilus*) *microplus*

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Abstract

The aim of this study was to assess the activity of aqueous (AE) and ethanolic extracts (EE) and pilocarpine hydrochloride, which were extracted and isolated from *Pilocarpus microphyllus* (Jaborandi), respectively, on *Rhipicephalus* (*Boophilus*) *microplus*. High performance liquid chromatography (HPLC) was performed to quantify these compounds. Larval packet and adult immersion tests were conducted with different concentrations. Five AE and EE concentrations, ranging from 6.2 to 100.0 mg mL^{-1}, and six concentrations of pilocarpine hydrochloride, ranging from 0.7 to 24.0 mg mL^{-1}, were tested. The lethal concentration (LC_{50}) of each extract for larvae and engorged females was calculated through Probit analysis. The concentration of pilocarpine hydrochloride obtained from the EE and the AE was 1.3 and 0.3% (*m/m*), respectively. Pilocarpine hydrochloride presented the highest acaricidal activity on larvae (LC_{50} 2.6 mg mL^{-1}) and engorged females (LC_{50} 11.8 mg mL^{-1}) of *R.*(*B.*) *microplus*, followed by the EE which presented LC_{50} of 56.4 and 15.9 mg mL^{-1}, for larvae and engorged females, respectively. Such results indicate that pilocarpine hydrochloride has acaricidal activity, and may be the primary compound responsible for this activity by *P. microphyllus* EE.

Keywords: Acaricide, tick, control, pilocarpine hydrochloride, *Pilocarpus micropyllus.*

Resumo

O objetivo desse estudo foi avaliar a atividade dos extratos aquoso (AE) e etanólico (EE) e do cloridrato de pilocarpina, que foram, respectivamente, extraídos e isolado de *Pilocarpus microphyllus* (Jaborandi), sobre *Rhipicephalus* (*Boophilus*) *microplus*. Cromatografia líquida de alta eficiência foi realizada para quantificação dos compostos. Testes de pacote de larvas e de imersão de adultos foram realizados com diferentes concentrações. Cinco concentrações do AE e EE variando de 6,2 a 100,0 mg mL^{-1} e seis concentrações do cloridrato de pilocarpina variando de 0,7 a 24,0 mg mL^{-1} foram testadas. A concentração letal (CL_{50}) de cada extrato para larvas e fêmeas ingurgitadas foi estimada por meio da análise Probit. A concentração de cloridrato de pilocarpina obtida do EE e AE foi de 1,3 e 0,3% (m/m), respectivamente. O cloridrato de pilocarpina apresentou a maior atividade carrapaticida sobre larvas (CL_{50} 2,6 mg mL^{-1}) e fêmeas ingurgitadas (CL_{50} 11,8 mg mL^{-1}) de *R.* (*B.*) *microplus*, seguido do EE que apresentou CL_{50} de 56,4 e 15,9 mg mL^{-1}, para larvas e fêmeas ingurgitadas, respectivamente. Tais resultados indicam que o cloridrato de pilocarpina apresenta atividade carrapaticida e pode ser o principal responsável pela atividade acaricida do EE de *P. microphyllus.*

Palavras-chave: Acaricida, carrapato, controle, cloridrato de pilocarpina, *Pilocarpus micropyllus*.

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Introduction

Rhipicephalus (*Boophilus*) *microplus* is the main cattle tick throughout the tropics (GRISI et al., 2014). Infestation by this parasite results in losses, mainly due to the decreased milk and meat production, and also due to the transmission of pathogens, which cause babesiosis and anaplasmosis (PETER et al., 2005; GRISI et al., 2014).

The control of *R.* (*B.*) *microplus* is conducted through chemical acaricides, and this process is often performed indiscriminately (FURLONG et al., 2003). The indiscriminate use of these products has contributed to the contamination of the environment and products of animal origin such as milk and meat. It has also led to the selection of ticks that are resistant to all active compounds that are normally found in the market (CASTRO-JANER et al., 2010; MILLER et al., 2013). In many countries, Brazil included, the acaricide-resistant tick population has been increasing, to the point that few acaricides have efficacy of more than 75% of the tick population (GRAF et al., 2004; MENDES et al., 2011). On the other hand, the results of certain studies suggest that environmentally friendly acaricides can be obtained from plants (GHOSH et al., 2011; GARCIA et al., 2012). As an alternative, plant extracts are used to manage animal health and parasite control in several regions in the world (GITHIORI et al., 2006; TAMIRU et al., 2013; NYAHANGARE et al., 2015).

Currently, several experimental studies have been assessing the anti-parasitic activity of medicinal plants (BORGES et al., 2011; DOMINGUES et al., 2013; LIMA et al., 2014). However, few plant extracts or compounds are produced on an industrial scale, which would enable their use for the control of ticks in the field.

The therapeutic use of Jaborandi (*Pilocarpus* sp.) has long been known by indigenous tribes from northern Brazil as an antidote for venoms and toxins. Currently, this medicinal plant is intensively used in the pharmaceutical industry (VITAL & ACCO, 2006). The genus *Pilocarpus* belongs to the family Rutaceae and is distributed in Brazil from the Amazon region as far as Rio Grande do Sul state (JOSEPH, 1967). This genus has many species of economic and medical importance, among which is *Pilocarpus microphyllus* (CORRÊA, 1969). Over the last three decades this specie has been considered one of the most important in the Brazilian flora for the production of medications. There are approximately 15 million of Jaborandi plants in cultivation and these produce about 10,000 kg of leaves per year (PINHEIRO, 2002, 2006). It is important to note that *P. microphyllus* has alkaloids that are viewed as promising sources in the quest for anti-parasitic agents (LEITE et al., 2011; VÉRAS et al., 2012).

Therefore, this study aimed at investigating the effects of aqueous and ethanolic extracts and pilocarpine hydrochloride obtained from *P. microphyllus* on the larvae and engorged females of the *R.*(*B.*) *microplus* tick.

Materials and Methods

Plant material

Leaves of *P. microphyllus* were collected at Anidro do Brasil Extrações S.A. pharmaceutical chemical company, located in the city of Parnaíba, Piauí, Brazil. Reference plant specimens were deposited in the Herbarium (CEN) of the *Embrapa Recursos Genéticos e Biotecnologia*, under registration number 81.098.

The leaves were sun dried until they reached ≤ 15% moisture content, and ground in a knife mill, so the aqueous (AE) and ethanolic (EE) extracts of *P. microphyllus* could be prepared. The AE was obtained through a 20-minute infusion with distilled water at 95 °C. The EE was obtained through maceration in ethanol (analytical grade) for seven days, with solvent changes. This material was filtered and concentrated in a rotary evaporator, and then diluted in 50% ethanol and 3% DMSO. Pilocarpine hydrochloride (100% purity) from *P. microphyllus* was kindly donated by Anidro do Brasil Extrações S.A. company (Parnaíba, PI, Brazil).

Chemical analysis

The pilocarpine assay % (*m/m*) in the AE and EE was determined through high performance liquid chromatography (HPLC) with a Merck-Hitachi Lachron Elite L-2000 chromatograph; mobile phase: 5% KH_2PO_4 and pH 2.5; Merck/ Lichrospher® 60 RP select B chromatography column (250 × 4.6 mm, 5 µm); 50 °C oven**;** UV 216 nm detector; 1 mL/min. flow; external standard of pilocarpine hydrochloride as the reference (VÉRAS et al., 2013).

Tick preparation

Engorged *R.* (*B.*) *microplus* females were collected from naturally infested calves. They were washed with water and dried with paper towels. A number of the engorged females were kept in a BOD chamber (27 °C and RH > 80%) until oviposition of the larvae (for the larval packet test) was finished. Previously, acaricide tests were performed which demonstrated resistance of the tick population to amidinic and pyrethroid compounds (personal information).

Preparation of the dilutions

The AE and the EE were prepared at concentrations of 6.2, 12.5, 25.0, 50.0 and 100.0 mg mL^{-1} and pilocarpine hydrochloride at concentrations of 0.7, 1.5, 3.0, 6.0, 12.0 and 24.0 mg mL^{-1}. The negative controls consisted of the solvents used in AE and EE dilutions, which were ultrapure water (C1) and 50% ethanol + 3% DMSO (C2), respectively. As the positive control, a mixture of 0.18 mg mL^{-1} cypermethrin, 0.30 mg mL^{-1} chlorpyrifos, and 0.012 mg mL^{-1} citronellal (Colosso®, Ouro Fino, São Paulo) was used. This solution was diluted at a 0.125% concentration in ultrapure water. All tests were replicated three times.

Larval packet test

The larval packet test was used following the method of Stone & Haydock (1962) and the Food and Agriculture Organization of the United Nations (FAO, 1971). Approximately 100 14-to-21-day-old larvae were placed between two filter papers (2 × 2 cm) forming a sandwich, after which they were impregnated with the treatments. Each "sandwich" was introduced in a filter paper envelope, and

then sealed, identified, and incubated at 27 °C with RH > 80% (LEITE, 1988). Living and dead larvae were counted 24 hours later, and mortality was calculated from the arithmetic average of three replicates.

Adult immersion test

The adult immersion test of engorged *R.* (*B.*) *microplus* females was conducted according to the method described by Drummond et al. (1973). After they were selected based on their mobility, body integrity, and size (≥ 4.5 mm), engorged females were weighed and distributed in ten-specimen groups with similar weights. The weight of engorged females ranging between 170 to 210 mg (BENNETT, 1974). The groups of females were immersed in solutions containing the several treatments for 5 minutes, and then dried in paper towels and stored in a chamber at 27 °C and RH > 80% for 18 days. After that period, the egg mass was weighed, transferred to adapted syringes, and incubated for 20 days (27 °C and RH > 80%). Hatchability was estimated from the average number of eggs and larvae. The egg production index (EPI), oviposition reduction (OR), reproductive efficiency (RE), and product effectiveness (PE) were calculated according to the following formulas: EPI = (weight of eggs/engorged females) × 100 (BENNETT, 1974); OR = (control EPI – treated EPI/control EPI) × 100 (ROULSTON et al., 1968); RE= (Egg mass weight × % of eclosion/weight of the mass of females) × 20,000; and PE = (control RE – treated RE)/(control RE × 100) (DRUMMOND et al., 1973).

This study was performed with the approval of the Ethics Committee for Animal Experimentation of the Federal University of Maranhão under approval number 23115018061.

Statistical analysis

The lethal concentration to kill 50% of the studied population (LC_{50}) of each extract for larvae and engorged females was calculated through Probit analysis with GraphPad Prism 6.0 software. The extract was considered to be significantly different from another one when the 95% confidence interval of LC_{50} was not overlapped (RODITAKIS et al., 2005). The differences among the concentrations of mortality against larvae and engorged females and the index RO, hatchability and EP were analyzed by F test of ANOVA followed by Tukey test ($p<0.05$).

Results and Discussion

The concentrations of pilocarpine obtained from the EE and the AE were 1.3 and 0.3% (*m/m*), respectively. The presence of active substances in the plant, even in small proportions or associated to other substances also in small proportions, may be responsible for the biological activity of some extracts (LIMA et al., 2014).

In this study, the EE at 100 mg mL^{-1} resulted in a 30.3% mortality for *R.* (*B.*) *microplus* larvae (Table 1). To increase

Table 1. Action of the aqueous (AE) and etanólico (EE) extract of *Pilocarpus microphyllus* and of pilocarpine hydrochloride (PC) on larvae (mortality) and engorged female (reduction of oviposition, hatchability and efficacy of the product) of *Rhipicephalus* (*Boophilus*) *microplus*.

Treatment	Concentration (mg mL^{-1})	Larvae	Engorged Females				
		Mortality (%)	Weight of females (g)	Weight of eggs (g)	Reduction of oviposition (%)	Hatchability (%)	Efficacy of the product (%)
C1	-	0.7±1.3^{a}	1.948±0.027	1.068±0.092	-	95.3±1.1^{a}	-
C2	-	0.0±0.0^{a}	1.887±0.018	1.131±0.025	-	96.4±1.2^{a}	-
AE	6.2	0.0±0.0^{a}	1.923±0.009	1.153±0.007	0.9±0.5^{a}	94.0±3.6^{a}	3.4±3.3^{a}
	12.5	0.0±0.0^{a}	1.922±0.020	1.135±0.025	2.4±1.1^{a}	94.9±0.5^{a}	3.9±0.6^{a}
	25.0	0.0±0.0^{a}	1.916±0.017	1.118±0.037	3.5±2.8^{a}	96.2±0.3^{a}	3.8±2.5^{a}
	50.0	0.0±0.0^{a}	1.925±0.017	1.140±0.024	2.1±2.6^{a}	96.2±1.7^{a}	2.3±1.6^{a}
	100.0	1.0±1.7^{a}	1.925±0.008	1.157±0.004	0.7±0.2^{a}	94.3±1.2^{a}	2.9±1.4^{a}
EE	6.2	1.5±1.7^{a}	1.891±0.002	1.000±0.023	11.8±2.1^{a,b}	92.3±2.5^{a}	14.5±1.8^{a,b}
	12.5	2.6±2.1^{a}	1.877±0.014	0.842±0.092	25.1±8.5^{b}	94.2±1.6^{a}	26.1±7.1^{b,c}
	25.0	3.5±2.3^{a}	1.870±0.008	0.378±0.113	66.3±10.0^{c}	91.3±2.9^{a}	67.6±10.4^{d}
	50.0	9.4±1.4^{a}	1.868±0.018	0.377±0.038	66.4±3.4^{c}	87.8±4.2^{a,b}	68.9±4.0^{d}
	100.0	30.3±8.3^{b}	1.887±0.010	0.268±0.157	76.3±13.9^{d}	85.5±5.3^{a,b}	79.2±11.4^{d,e}
PC	0.7	10.2±6.1^{a}	1.956±0.009	1.015±0.078	7.2±6.9^{a}	93.7±3.4^{a}	10.4±3.3^{a,b}
	1.5	32.7±14.8^{b}	1.962±0.029	1.028±0.049	6.2±5.8^{a}	90.7±0.9^{a}	12.4±4.7^{a,b}
	3.0	58.8±9.0^{c}	1.983±0.007	1.017±0.033	8.2±3.2^{a}	95.5±1.4^{a}	9.6±2.1^{a,b}
	6.0	88.4±7.6^{d}	1.930±0.025	0.947±0.111	12.3±9.4^{a,b}	75.6±6.0^{b}	31.4±11.6^{b,c}
	12.0	99.0±0.9^{d}	1.980±0.009	0.666±0.116	30.4±12.3^{b}	74.8±13.8^{b}	43.2±19.1^{c}
	24.0	96.6±2.1^{d}	1.978±0.014	0.219±0.037	77.1±3.7^{c,d}	26.8±6.8^{c}	93.2±2.8^{e}

Values represent average ± standard deviation; C1: Negative control (mili-q water); C2: Negative control (ethanol 50% + DMSO 3%); Means with the same letter, for each treatment, are not statistically different ($p \leq 0.05$).

mortality, higher concentrations would be necessary making its use difficult in the field (LC_{50} 56.4 mg mL^{-1}; 95% CI 38.98 – 81.66) (Table 2). In regards to the *R.* (*B.*) *microplus* engorged females, the efficiency of EE at 100 mg mL^{-1} was 79.2% (LC_{50}15.9 mg mL^{-1}; 95% CI 14.30-17.88), which demonstrates a significant effect on their reproductive capabilities (Table 1). This effect is mainly due to reduced oviposition (76.3%). There was no significant influence on the hatchability of eggs (85.5%) (Table 1). When the AE was used against larvae and engorged *R.* (*B.*) *microplus* females, no parameter was significantly different, and no acaricidal activity was found (Table 1).

The assessment of pilocarpine hydrochloride on larvae obtained a maximum mortality (99.0%) (Table 1) at 12 mg mL^{-1} (LC_{50} 2.6 mg mL^{-1}; 95% IC 2.36-2.97) (Table 2). At 24 mg mL^{-1}, pilocarpine hydrochloride was found to be highly effective (93.2%) in its effect on engorged females, (LC_{50} 11.8 mg mL^{-1}; 95% IC 9.60-14.41). This was due to low oviposition (77.1%) and to low hatchability (26.8%) (Table 1), which demonstrates the reduced egg fertility resulting from reproductive impairment. The number of offspring was consequently reduced.

In this study, the effect of isolated pilocarpine hydrochloride on *R.* (*B.*) *microplus* larvae and engorged females was found to be higher than the effect of the EE of *P. microphyllus*, which contained 1.3% of the compound. In contrast, Chungsamarnyart & Jansawan (1996), when assessing other species in the Rutaceae family, observed a significantly reduced effect of the d-limonene constituent on *R.* (*B.*) *microplus* larvae and females, compared to the effect of oils from *Citrus* spp. species, from which it originated, indicating, in that case, a possible synergy among its compounds.

The effect of pilocarpine hydrochloride was shown to be similar to that of the positive control (a mixture of cypermethrin, chlorpyrifos, and citronellal) on larvae (mortality of 100%) in the last three concentrations (6, 12 and 24 mg mL^{-1}) and engorged females (efficacy of 98,2%) in the last concentration (24 mg mL^{-1}) for the three parameters (reduction of oviposition, hatchability and efficacy of the product) (Table 1). These results indicate the potential of that substance in the control of *R.* (*B.*) *microplus*. An advantage in the use of plant extracts and their compounds is in the possibility of controlling cattle ticks in organic production systems, where the use of synthetic pesticides is prohibited. However, this is only true when these extracts do not cause adverse reactions to the host and workers, and do not leave residues in products of animal origin. It is also an alternative way to control resistant populations (BORGES et al., 2011).

Table 2. Lethal concentration of *Pilocarpus microphyllus* leaves ethanolic extract and of pilocarpine hydrochloride against *Rhipicephalus* (*Boophilus*) *microplus* larvae and engorged females.

Treatment	LC_{50} (mg mL^{-1})	IC 95%	R^2
Larvae			
Ethanolic extract	56.4[c]	38.98-81.66	0.90
pilocarpine hydrochloride	2.6[a]	2.36-2.97	0.97
Engorged female			
Ethanolic extract	15.9[b]	14.30-17.88	0.92
pilocarpine hydrochloride	11.8[b]	9.60-14.41	0.88

LC50 concentration (mg/mL) at which 50% of the *R.* (*B.*) *microplus* died; IC 95% - Confidence limits at 95% probability; R^2 = Coeficient of determination.

Synergistic combinations are also used to reduce the dosage of substances and the risk of parasites developing resistance (TRIPATHI et al., 2009). The effect is enhanced by the interaction of its various compounds (CHAGAS et al., 2002). The resistance develops more slowly when extracts or compounds are used, due to the mixture of active substances with different mechanisms (MULLA & SU, 1999). Therefore, an extract-based acaricide, when compared to one composed of a single main substance, has an edge in terms of both reduced resistance and cost.

P. microphyllus, from which pilocarpine is extracted, is native and widely spread in Piauí, Maranhão and Pará states of Brazil (PINHEIRO, 1997). Currently, the annual industrial production of Jaborandi leaves is 10,000 kg, of which approximately 1% is pilocarpine (VÉRAS et al., 2013) demonstrating the production potential of this medicinal species and its pilocarpine alkaloid. It is extremely important to emphasize that Brazil is the only supplier of this active pharmaceutical ingredient (API) for the international pharmaceutical industry (BRANDÃO et al., 2008) and studies with *P. microphyllus* alkaloids and its industrial applications are of fundamental importance for a large number of economically precarious communities whose main source of income is the species harvest in underdeveloped regions of northeastern Brazil (LIMA et. al., 2015).

Pilocarpine is a cholinergic alkaloid that, when connected to muscarinic receptors, acts on the parasympathetic nervous system and results in smooth muscle contraction. Therefore, it stimulates secretory glands, relaxes sphincters, stimulates peristalsis, and influences blood pressure in humans (PAPPANO, 2006). There is evidence that muscarinic receptors are present in the synganglion of *R.* (*B.*) *microplus* (TURBERG et al., 1996). Peripheral nerves emerge from the synganglion, during the several life stages of ticks, and they are responsible for the innervation of muscles, sensory structures, tegmen, and several internal organs (SONENSHINE, 1991). Pilocarpine may have inhibited engorged female reproduction and killed the larvae, as observed in this study, due to the excessive stimulation of smooth muscles in digestive, reproduction, or circulatory organs, thus producing a physiological imbalance in *R.* (*B.*) *microplus*.

Pilocarpine is also the most widely used agonist to induce salivation in ticks, and the salive obtained is used to develop a vaccine against them (RIBEIRO et al., 2004). However, no previous study has reported pilocarpine leading to the inhibition of the reproduction of females or the mortality of *R.* (*B.*) *microplus* larvae.

Our results indicate that the EE of *P. microphyllus* has significant effects on *R.* (*B.*) *microplus* larvae and engorged females and that pilocarpine hydrochloride may be one of the main agents responsible for these effects. Based on our results, we infer that higher pilocarpine contents in the EE may enhance the effects obtained on *R.* (*B.*) *microplus*. The use of certain processes to optimize extraction of the *P. microphyllus* EE may result in the possibly more efficient recovery of extracts with higher concentrations of pilocarpine alkaloid. The acidification of *P. microphyllus* leaves and the further extraction with polar solvents such as water and ethanol favor the

extraction of total alkaloids that can be subsequently submitted to fractionation and prepurification processes (VÉRAS et al., 2013). Due to the high cost of obtaining isolated pilocarpine hydrochloride, obtaining extracts with a higher content of that alkaloid is an alternative that could possibly be used as a low-cost acaricide. Complementary studies should be performed to establish the safety of the use of these extracts in animals.

Conclusion

The ethanolic extracts of *P. microphyllus* and pilocarpine hydrochloride have significant effects on *R.* (*B.*) *microplus* larvae and, most notably, on engorged females. These results suggest the possibility of using these extracts as acaricides, conducting new studies with optimized extracts and fractions of *P. microphyllus* with a higher pilocarpine content and with the goal of enhancing its effects on *R.* (*B.*) *microplus*. Furthermore, studies that add potential biotechnological value to this species will benefit underdeveloped communities whose main source of income is the harvest of leaves from native species in the states of Piauí, Maranhão and Pará in northeast Brazil.

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