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Original Article

In vitro acaricidal activity of different ectoparasiticide classes against *Amblyomma sculptum* larvae

Atividade acaricida *in vitro* de diferentes classes de ectoparasiticidas frente a larvas de *Amblyomma sculptum*

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Abstract

Zoonoses are major causes of morbidity and mortality worldwide. Among them, Brazilian Spotted Fever (BSF) is an important one that occurs in some regions of South America and can be transmitted by the "star tick" *Amblyomma sculptum*. Application of acaricides against the larval stage is important as strategy of population control. However, there is still a deficiency of studies on chemical control of *A. sculptum* and the present work aims to evaluate the *in vitro* acaricidal activity of cypermethrin, flumethrin, deltamethrin, fipronil, coumaphos and chlorpyrifos against *A. sculptum* larvae. Bioassays were performed using the larval immersion test method. A discriminatory analysis between the antiparasitic classes most used for tick control was carried out, which made it possible to determine the classes with higher potential for controlling *A. sculptum* larvae. Our results showed that *A. sculptum* larvae present highest sensitivity to the synthetic pyrethroid group, followed by the phenylpyrazole, organophosphate and macrocyclic lactone groups. These findings may support studies on improvement of tick control as in animals as in the environment.

Keywords: Star tick, tick control, *in vitro* assay.

Resumo

As zoonoses são a maior causa de morbidade de mortalidade no mundo. A Febre Maculosa Brasileira (FMB) é uma importante zoonose que ocorre em algumas regiões da América do Sul e pode ser transmitida pelo "carrapato-estrela" *Amblyomma sculptum*. A aplicação de acaricidas, frente ao estágio larval, é importante como estratégia no controle da população. No entanto, ainda há uma deficiência de estudos para o controle químico de *A. sculptum*. Devido à necessidade de mais informações sobre o controle de *A. sculptum,* o presente trabalho tem como objetivo avaliar a atividade acaricida *in vitro* de cipermetrina, flumetrina, deltametrina, fipronil, coumafós e clorpirifós frente a larvas de *A. sculptum*. Os bioensaios foram realizados pelo método Teste de Imersão de Larva. Foi realizada uma análise discriminatória entre as classes antiparasitárias mais utilizadas para controle de carrapatos, possibilitando determinar classes com maior potencial para o controle de larvas de *A. sculptum*. Os resultados deste trabalho mostraram que as larvas de *A. sculptum* apresentam maior sensibilidade ao grupo dos piretroides sintéticos, seguido pelos grupos fenilpirazóis, organofosforados e lactonas macrocíclicas. Esses achados poderiam apoiar estudos visando ao controle do carrapato tanto em animais quanto no meio ambiente.

Palavras-chave: Carrapato-estrela, controle de carrapatos, ensaio *in vitro.*

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Introduction

Ticks are widely distributed in Brazil with 70 species, being the *Amblyomma* genera (32 spp.) the most representative (Dantas-Torres et al., 2019). They need to feed on the blood of vertebrates (mainly mammals) and are responsible for causing cutaneous lesions, anemia, inoculation of toxins and transmission of pathogens. These occurrences can lead to host death (Prata, 2005; Rodrigues et al., 2015; Moraes-Filho, 2017). *Amblyomma sculptum* Berlese, during a long time cited as *Amblyomma cajennense* (Fabricius), also called by "star tick", the most important tick species, given that it has the capacity to parasitize several animal species, including humans. Infected ticks can transmit the bacterium *Rickettsia rickettsii*, etiological agent of Brazilian Spotted Fever (BSF), an important zoonosis and the most lethal rickettsiosis in the world (Bechah et al., 2008; Labruna et al., 2002; Labruna, 2009).

Amblyomma sculptum is part of a complex of six species (*A. cajennense sensu stricto s.s.*, *Amblyomma mixtum* Koch, *A. sculptum*, *Amblyomma tonelliae* Nava, Beati & Labruna, *Amblyomma interandinum* Beati, Nava & Cáceres and *Amblyomma patinoi* Labruna, Nava & Beati), named *Amblyomma cajennense* complex. Each of the species reported has a distinct geographical distribution. The species *A. sculptum* is commonly found in Argentina, Bolivia, Paraguay and peri-Amazonian areas of Brazil (in the states of Rio de Janeiro, Espírito Santo, Minas Gerais, São Paulo, Paraná, Pernambuco, Piauí, Mato Grosso, Mato Grosso do Sul and Goiás). Thus, previous occurrences of "star ticks" reported as *A. cajennense* causing BSF in these regions of Brazil were probably due to *A. sculptum* (Nava et al., 2014).

Although *A. sculptum* has low parasitic specificity, especially in the immature phases, capybaras and horses are the preferred hosts for all stages of this species (Labruna et al., 2001). For effective control over BSF, strategic tick control could be useful, in order to reduce the number of ectoparasites both among animals and in the environment. The immature stages of ticks are more sensitive to acaricides than is the adult stage. Therefore, reduction of the immature tick population consequently provides a reduction in the number of adults (Rodrigues et al., 2015). The main classes of acaricides currently used in Brazil, via the topical route, are macrocyclic lactones, phenylpyrazoles, pyrethroids and organophosphates. However, there is still a deficiency of studies on chemical control over *A. sculptum.*

Given the need for more information about *A. sculptum* control and to enable and ensure the efficacy and safety of the use of active ingredients for controlling ticks, it is first necessary to determine the effective concentrations. Evaluation of *in vitro* activity and estimation of LC_{50} and LC_{90} values are important tools at this stage. Therefore, the aim of the present study was to evaluate the *in vitro* acaricidal activity of cypermethrin, flumethrin, deltamethrin, fipronil, coumaphos and chlorpyrifos against *A. sculptum* larvae.

Material and methods

Reagents and chemicals

Technical-grade reagents were purchased as follows: acetone and Triton-X from Vetec® (Duque de Caxias, BR), N-methylpyrrolidone (NMP) and ethanol from Synth® (Diadema, BR) and xylene from Isofar® (Duque de Caxias, BR). The technical-grade active ingredients cypermethrin (93.1%), flumethrin (97.4%) and fipronil (99.2%) were provided by CEVA® (Paulínia, BR). Technical-grade coumaphos (98.1%), chlorpyrifos (98.7%) and ivermectin (98.7%) were provided by Champion® (Campinas, BR). Butox® MSD (deltamethrin) was purchased from a local market.

Preparation of test solutions

The emulsifiable concentrates of each technical-grade active ingredient were prepared as described in Table 1. The surfactant Triton-x was added to all the emulsifiable concentrates at 2%. Different solvents (ethanol, N-methylpyrrolidone, xylene and acetone) were used to ensure complete solubilization of the active ingredients. Stock solutions were prepared through dilutions (1:100) of the emulsifiable concentrates in pure water. Working concentrations (n = 10) were prepared using the diluents at the concentration range described in Table 1. The test solutions were prepared in accordance with the recommendations of the FAO Guidelines for Resistance Management and Integrated Parasite Control in Ruminants (FAO, 2004).

In vitro larvicidal assay

The experiments followed the standards established by the Ethics Committee for Animal Use (CEUA) of the Veterinary Institute, Federal Rural University of Rio de Janeiro (UFRRJ). The larvae of *A. sculptum* (CEUA/IV no. 7699190418) that were used in the experiment were obtained from colonies maintained in rabbits in the Laboratory for Experimental Chemotherapy in Veterinary Parasitology of UFRRJ.

Table 1. Preparation of emulsifiable concentrates and test solutions of active ingredients for *in vitro* larvicidal assays.

Class	Active	Emulsifiable concentrate**		Stock solution***	Diluent composition ****	Concentration range (μg.mL^{-1})
		% AI	Vehicle	% AI		
ML	Ivermectin	5	EtOH	0.05	1% EtOH	0.1 - 10.000
OP	Coumaphos	1	NMP	0.010	1% NMP	1 - 100
	Chlorpyrifos	20	50% acetone 50% xylene	0.20	0.50% acetone 0.50% xylene	5 - 1250
PYZ	Fipronil	1	Acetone	0.010	1% acetone	1 - 60
SP	Cypermethrin	5	50% acetone 50% xylene	0.050	0.50% acetone 0.50% xylene	5 - 50
	Flumethrin	0.10		0.001		0.0012 - 0.62
	Deltamethrin*	2.5		0.025	0.02% Triton	0.50 - 20

ML: Macrocyclic Lactones; OP: Organophosphates; PYZ: Phenylpyrazole; SP: Synthetic Pyrethroids; NMP: N-methylpyrrolidone; AI: Active Ingredient; EtOH: ethanol; NMP: N-methyl-pyrrolidone. *Commercial product Butox®; **Triton-x added at 2%; ***Prepared in pure water; ****Triton-x 0.02% in pure water.

The colony of *A. sculptum* was created from fed females collected from horses in the UFRRJ herd that did not receive any carrapaticidal treatment in the last 6 months. For the *in vitro assay,* 12th generation larvae were used.

The bioassays were performed using the larval immersion test (LIT) method (Shaw, 1966), as adapted by Leite (1988) and Chagas et al. (2002). For each concentration, approximately 100 non-fed 35-day-old larvae of *A. sculptum* were deposited on a 2 cm x 2 cm filter paper sandwich, which was impregnated with 0.5 mL of the test solution. The filter paper sandwich was wrapped in a filter paper envelope (6 cm x 6 cm) that was then properly sealed with binder clips. The envelopes were kept in a climatized chamber at 27 ± 1 °C and relative humidity of 80 ± 10%. The mortality assessment was performed after 24 hours for organophosphates, phenylpyrazole and synthetic pyrethroids and after 48 hours for macrocyclic lactones (FAO, 2004). The evaluation criterion used was motility, i.e. any larva that presented minimal movement was considered alive. The mean number of live larvae per concentration was evaluated with the aid of a stereomicroscope. The tests were performed in duplicate for each concentration. Mortality was calculated in accordance with the following formula proposed by Abbott (1925): Mortality (%) = dead larvae x 100 / total larvae.

Statistical analysis

The Probit analysis method was used to assess LC_{50} and LC_{90} lethal concentration values together with their 95% confidence interval (95% CI) (μg.mL^{-1}) and the slope ± SE of the concentration curve, with the χ^2 test to determine the accuracy of data fitting. The goodness-of-fit test showed that the values did not present any significant heterogeneity at the level of $p \geq 0.05$. The Probit analysis estimates were calculated using the IBM SPSS statistical software, version 23.

Results

Mortality

The bioassay results showed that all the active ingredients tested exhibited acaricidal activity against *A. sculptum* larvae (Table 2). The best efficacy results were found for the synthetic pyrethroid group, which achieved 100% efficacy at the concentrations of 0.625 μg.mL^{-1}, 5 μg.mL^{-1} and 20 μg.mL^{-1}, for flumethrin, deltamethrin and cypermethrin respectively. Organophosphates and phenylpyrazole also presented good results with 100% efficacy at 500 μg.mL^{-1}, 75 μg.mL^{-1} and 60 μg.mL^{-1}, for chlorpyrifos, coumaphos and fipronil respectively. Ivermectin achieved 100% efficacy at the highest concentration (5500 μg.mL^{-1}).

Table 2. *In vitro* activity through larvae immersion test (% mortality) against larvae of *Amblyomma sculptum*.

Chlorpyrifos		Coumaphos		Cypermethrin		Flumethrin		Deltamethrin		Fipronil		Ivermectin	
Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%
5	0	1	0	5	0	0.001	0	0.5	3	1	0	0.1	0
10	0	2.5	0	6	5	0.002	0	1	13	2.5	29	0.5	0
50	70	5	5	7	36	0.005	0	2	75	5	31	1.0	0
100	80	7.5	10	8	92	0.010	0	3	94	7.5	41	5.5	0
250	95	10	67	9	97	0.020	30	4	99	10	89	10	0
350	97	20	88	10	98	0.039	26	5	100	20	99	55	10
500	100	35	95	20	100	0.078	59	8	97	30	98	100	35
750	100	50	91	30	100	0.156	85	10	98	40	99	550	84
1000	100	75	100	40	100	0.313	93	15	100	50	98	1000	93
1250	99	100	99	50	100	0.625	100	20	100	60	100	5500	100

Conc.: Concentration of active ingredients expressed in $\mu g.mL^{-1}$; %: Mortality (%) among *Amblyomma sculptum* larvae after 24 hours for chlorpyrifos, coumaphos, cypermethrin, flumethrin, deltamethrin and fipronil and after 48 hours for ivermectin.

LC_{50} and LC_{90} estimates

The LC_{50} and LC_{90} values for the active ingredients evaluated, against *A. sculptum* larvae, are described in Figure 1. The LC_{50} ranged from 0.06 $\mu g.mL^{-1}$ to 187.275 $\mu g.mL^{-1}$, while the LC_{90} ranged from 0.216 $\mu g.mL^{-1}$ to 711.084 $\mu g.mL^{-1}$. The linear regression coefficient (r) was higher than 0.85 and the p-value of the goodness-of-fit test was higher than 0.05 for all the active ingredients evaluated. This demonstrated through the Probit analysis that the data fitting was accurate, without any significant heterogeneity (Table 3).

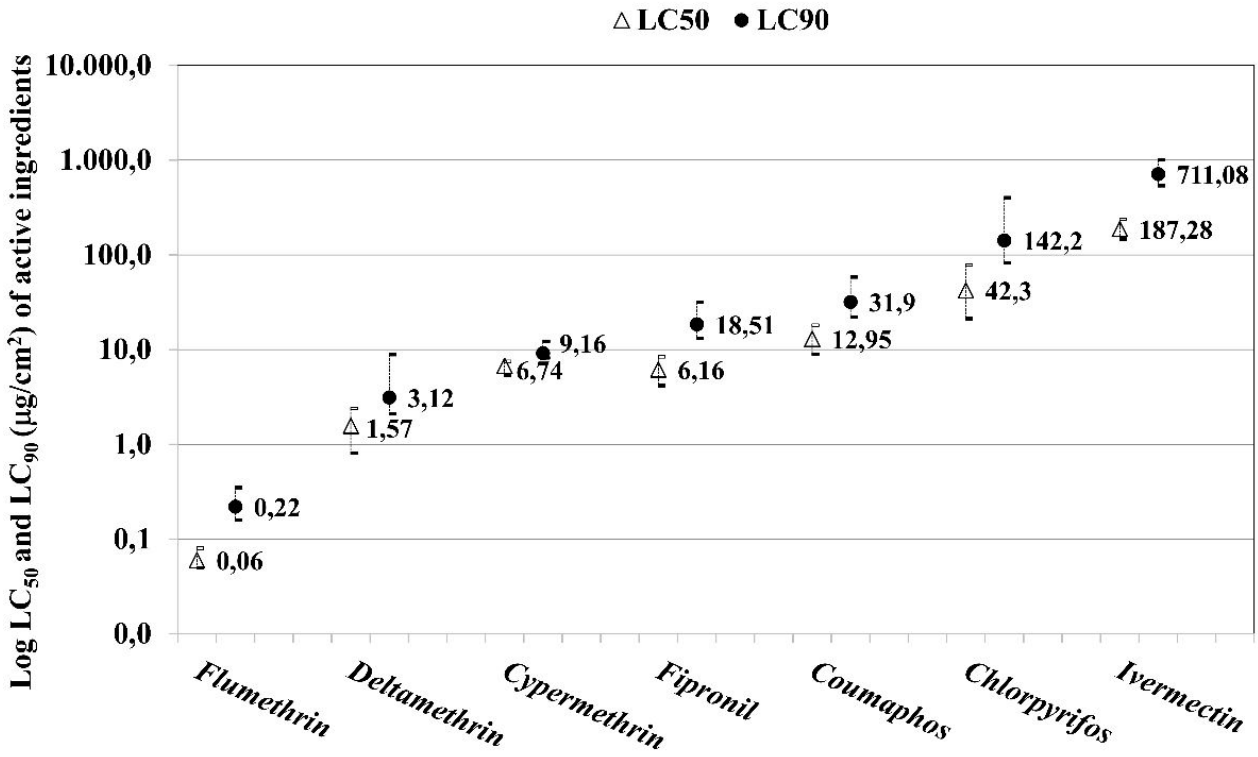

Figure 1. LC_{50} and LC_{90} values for active ingredients evaluated against *Amblyomma sculptum* larvae.

Table 3. Probit analyses on mortality data obtained from bioassays against larvae of *Amblyomma sculptum.*

	LC_{50} (μg.mL^{-1}) (95% CI)	LC_{90} (μg. mL^{-1}) (95% CI)	Slope (SE)	r	χ^2
Flumethrin	0.060 (0.039- 0.075)	0.231 (0.164-0.395)	1.89 (0.107)	0.962	0.055
Deltamethrin	1.568 (0.812-2.395)	3.116 (2.085-8.849)	4.296 (0.213)	0.882	0.085
Cypermethrin	7.080 (3.367-8.097)	8.243 (7.255-19.285)	19.402 (1.504)	0.929	0.094
Coumaphos	12.951 (8.988-18.134)	31.875 (22.289-58.236)	3.276 (0.171)	0.869	0.071
Chlorpyrifos	45.251 (21.210-78.135)	142.222 (81.99-401.407)	2.577 (0.159)	0.980	0.092
Ivermectin	187.275 (147.277-236.652)	711.084 (536.487-1013.40)	2.212 (0.193)	0.987	0.977
Fipronil	6.157 (4.174-8.378)	18.514 (13.170-31.599)	2.68 (0.12)	0.856	0.083

Probit analyses were performed for all data using the IBM SPSS statistical software, version 23. Data are given as 50% (LC_{50}) and 90% (LC_{90}) lethal concentration values together with their 95% confidence interval (95% CI) (μg.mL^{-1}); the slope ± SE of the concentration curve; and the χ^2 test as the accuracy of data fitting to the Probit analysis (goodness-of-fit test: values did not show any significant heterogeneity at the level of $p \geq 0.05$).

Discussion

The scarcity of studies for the control of *A. sculptum* is perhaps one of the reasons that in the last 30 years few products have been launched with instructions for use in horses, with the use of off-label products in these animals being common. Labruna et al. (2004) reported that only a few pyrethroid formulations would be available for the control of *A. cajennense* in horses.

Our results showed that *A. sculptum* larvae presented highest sensitivity to the synthetic pyrethroid group, followed by the phenylpyrazole, organophosphate and macrocyclic lactone groups. These active groups present different modes of action, as follows: via the Gaba chloride channel for ivermectin and fipronil (Papich, 2012; Barros & Di Stasi, 2012); via the kinetics of sodium channels for pyrethroids (Adams, 2003); and via irreversible inhibitors of acetylcholinesterase for organophosphates (Barros & Di Stasi, 2012). These groups are widely used for tick control (Taylor, 2001), but there is a lack of knowledge about their activity against *A. sculptum*.

Bittencourt et al. (1989) evaluated *in vitro* non-fed larvae of *A. cajennense sensu lato* against different pyrethroids (deltamethrin, alfamethrin, flumethrin and fenvalerate) and, as in the present study, the authors also found that flumethrin obtained better results (LC_{50} = 0.85 μg.mL^{-1} and LC_{90} = 2.7 μg.mL^{-1}). The other pyrethroids showed larvicidal activity in higher concentrations: deltamethrin (LC_{50} = 2.2 μg.mL^{-1} and LC_{90} = 6.2 μg.mL^{-1}), alfamethrin (LC_{50} = 2.4 μg.mL^{-1} and LC_{90} = 23 μg.mL^{-1}) and fenvalerate (LC_{50} = 7.5 μg.mL^{-1} and LC_{90} = 80 μg.mL^{-1})

Among the pyrethroids evaluated in the present study, the larvicidal activity of flumethrin was most pronounced (LC_{50} = 0.06 μg.mL^{-1} and LC_{90} = 0.23 μg.mL^{-1}) lower than that observed by Bittencourt et al. (1989), with relative potency that was up to 13-fold and 35-fold higher than those of deltamethrin (LC_{50} = 1.56 μg.mL^{-1} and LC_{90} = 3.11 μg mL^{-1}) and cypermethrin (LC_{50} = 7.08 μg.mL^{-1} and LC_{90} = 8.24 μg.mL^{-1}), respectively.

Ticks used in both studies (ours and Bittencourt et al., 1989) obtained their colonies from specimens collected from horses in the UFRRJ herd. After 30 years, the LC_{50} levels for flumethrin and deltamethrin do not increase, indicating that the susceptibility of this population over time has not undergone significant changes in sensitivity. A possible explanation for this fact in Brazil would be the use of fipronil off-label, since its commercial presentation is intended for use in cattle.

In an *in vitro* study conducted with non-fed larvae of *A. cajennense* in the state of Goiás, Brazil, Freitas et al. (2011) evaluated the susceptibility of a population to different ectoparasiticides: deltamethrin (LC_{50} = 0.03 μg.mL^{-1} and LC_{90} = 0.2 μg.mL^{-1}), permethrin (LC_{50} = 0.06 μg.mL^{-1} and LC_{90} = 1.15 μg. mL^{-1}), cypermethrin + piperonyl butoxide (PBO) (LC_{50} = 0.017 μg.mL^{-1} and LC_{90} = 0.81 μg.mL^{-1}) and amitraz (LC_{50} = 0.003 μg.mL^{-1} and LC_{90} = 3.6 μg.mL^{-1}). The LC_{50} of deltamethrin and cypermethrin obtained in the present study were higher than those observed by

Freitas et al. (2011). This fact may be related to a lower susceptibility of this population, however for cypermethrin it is important to note that the association with PBO may have caused a synergistic effect, promoting greater efficacy (Taylor, 2001).

Alonso-Díaz et al. (2013), carried out several *in vitro* studies in Mexico to determine the levels of susceptibility of populations of *A. cajennense* to different synthetic acaricides, observing high frequencies of resistance to organophosphates and amitraz. The authors highlight the need for more *in vitro* studies to be carried out to monitor susceptibility levels.

Despite the relevance that this parasite has for horses and human's health, there is still a lack of *in vitro* and *in vivo* studies to evaluate synthetic acaricides that can be used in the control of horse ticks. The determination of the acaricidal action of the active ingredients used in the present study against *A. sculptum* is relevant, as it indicates that all of them presented efficacy levels, however it is emphasized that the results found do not allow us to state that they can be used to control this tick.

The study was evaluated with non-fed larvae and as stated by Bittencourt et al. (1989), this stage is the one that presents the greatest susceptibility to pyrethroids. Other studies should be conducted with the other stages (not fed and fed) in order to be able to affirm that an active ingredient has high potential to be used in the control of *A. sculptum*. The bioassays with *A. sculptum* larvae can serve as initial tests for the selection of promising active ingredients, as well as for the monitoring of resistant populations.

Conclusion

The synthetic pyrethroids deltamethrin, cypermethrin and flumethrin and the phenylpyrazole fipronil demonstrated high larvicidal efficacy in *in vitro* assays against the larvae of *Amblyomma sculptum.*

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