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

Atividade acaricida *in vitro* de diferentes classes de ectoparasiticidas frente a *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.

**Resume**

As zoonoses são uma causa maior 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, coumáfos 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 piretroídeis 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, *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.
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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 macyclic 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 ≥ 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$^{-1}$).

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**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$^{-1}$) |
|---------|------------|-----------------------------|-------------------|-------------------------|-----------------------------------|
| ML      | Ivermectin | 5                           | 0.05              | 1% EtOH                 | 0.1 - 10.000                     |
| OP      | Coumaphos  | 1                           | 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                           | 0.010             | 1% acetone              | 1 - 60                           |
| SP      | Cypermethrin | 5                        | 0.050             | 0.50% acetone 0.50% xylene | 5 - 50                           |
| Flumethrin | 0.10       | 0.001                       | 0.0012 - 0.62     | 0.02% Triton            | 0.50 - 20                       |
| Deltamethrin* | 2.5       | 0.025                       | 0.02% Triton      | 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.
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The LC$_{50}$ and LC$_{90}$ values for the active ingredients evaluated, against _Amblyomma sculptum_ larvae, are described in Figure 1. The LC$_{50}$ ranged from 0.06 μg.mL$^{-1}$ to 187.275 μg.mL$^{-1}$, while the LC$_{90}$ ranged from 0.216 μg.mL$^{-1}$ to 711.084 μg.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).

### 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.      |
| 5     | 0            | 1         | 0            | 0.001      | 0            | 0.5      | 3          |
| 10    | 0            | 2.5       | 0            | 0.002      | 0            | 1        | 13         |
| 50    | 70           | 5         | 5            | 0.005      | 0            | 2        | 75         |
| 100   | 80           | 7.5       | 10           | 0.010      | 0            | 3        | 94         |
| 250   | 95           | 10        | 67           | 0.020      | 30           | 4        | 99         |
| 350   | 97           | 20        | 88           | 0.039      | 26           | 5        | 100        |
| 500   | 100          | 35        | 95           | 0.078      | 59           | 8        | 97         |
| 750   | 100          | 50        | 91           | 0.156      | 85           | 10       | 98         |
| 1000  | 100          | 75        | 100          | 0.313      | 93           | 15       | 100        |
| 1250  | 99           | 100       | 99           | 0.625      | 100          | 20       | 100        |
|       |              | 60        | 100          | 100        | 550          | 100      | 93         |

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

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.

|            | $\text{LC}_{50}$ (μg.mL$^{-1}$) (95% CI) | $\text{LC}_{90}$ (μg.mL$^{-1}$) (95% CI) | Slope (SE) | r | $\chi^2$
|------------|------------------------------------------|-----------------------------------------|------------|---|--------|
| 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 $50\%$ ($\text{LC}_{50}$) and $90\%$ ($\text{LC}_{90}$) lethal concentration values together with their $95\%$ confidence interval ($\mu$g.mL$^{-1}$); the slope ± SE of the concentration curve; and the $\chi^2$ 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 ($\text{LC}_{50} = 0.85 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 2.7 \mu$g.mL$^{-1}$). The other pyrethroids showed larvicidal activity in higher concentrations: deltamethrin ($\text{LC}_{50} = 2.2 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 6.2 \mu$g.mL$^{-1}$), alfamethrin ($\text{LC}_{50} = 2.4 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 23 \mu$g.mL$^{-1}$) and fenvalerate ($\text{LC}_{50} = 7.5 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 80 \mu$g.mL$^{-1}$).

Among the pyrethroids evaluated in the present study, the larvicidal activity of flumethrin was most pronounced ($\text{LC}_{50} = 0.06 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 0.23 \mu$g.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 ($\text{LC}_{50} = 1.56 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 3.11 \mu$g.mL$^{-1}$) and cypermethrin ($\text{LC}_{50} = 7.08 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 8.24 \mu$g.mL$^{-1}$), respectively.

Ticks used in both studies (ours and Bittencourt et al., 1989) obtained their colonies from specimens collected from horses in the UFRJR herd. After 30 years, the $\text{LC}_{50}$ 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 ($\text{LC}_{50} = 0.03 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 0.2 \mu$g.mL$^{-1}$), permethrin ($\text{LC}_{50} = 0.06 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 1.15 \mu$g.mL$^{-1}$), cypermethrin + piperonyl butoxide (PBO) ($\text{LC}_{50} = 0.017 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 0.81 \mu$g.mL$^{-1}$) and amitraz ($\text{LC}_{50} = 0.003 \mu$g.mL$^{-1}$ and $\text{LC}_{90} = 3.6 \mu$g.mL$^{-1}$). The $\text{LC}_{50}$ of deltamethrin and cypermethrin obtained in the present study were higher than those observed by
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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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