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Original Article

Anti-tick effect and cholinesterase inhibition caused by *Prosopis juliflora* alkaloids: *in vitro* and *in silico* studies

Efeito carrapaticida e inibição da acetilcolinesterase por alcaloides de *Prosopis juliflora*: estudos *in vitro* e *in silico*

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Abstract

We investigated the *in vitro* acaricide activity of the methanolic extract (ME) and alkaloid-rich fraction (AF) of *Prosopis juliflora* on *Rhipicephalus microplus* and correlated this effect with acetylcholinesterase (AChE) inhibition. The acaricide activity was evaluated using adult and larval immersion tests. Also, we studied the possible interaction mechanism of the major alkaloids present in this fraction via molecular docking at the active site of *R. microplus* AChE1 (*Rm*AChE1). Higher reproductive inhibitory activity of the AF was recorded, with effective concentration (EC_{50}) four times lower than that of the ME (31.6 versus 121 mg/mL). The AF caused mortality of tick larvae, with lethal concentration 50% (LC_{50}) of 13.8 mg/mL. Both ME and AF were seen to have anticholinesterase activity on AChE of *R. microplus* larvae, while AF was more active with half-maximal inhibitory concentration (IC_{50}) of 0.041 mg/mL. The LC-MS/MS analyses on the AF led to identification of three alkaloids: prosopine (**1**), juliprosinine (**2**) and juliprosopine (**3**). The molecular docking studies revealed that these alkaloids had interactions at the active site of the *Rm*AChE1, mainly relating to hydrogen bonds and cation-pi interactions. We concluded that the alkaloids of *P. juliflora* showed acaricide activity on *R. microplus* and acted through an anticholinesterase mechanism.

Keywords: Fabaceae, alkaloids, acaricide, anticholinesterase, molecular docking.

Resumo

A atividade carrapaticida *in vitro* do extrato metanólico (EM) e da fração de alcaloides (FA) de *Prosopis juliflora* foi investigada, frente ao *Rhipicephalus microplus,* e relacionada com a inibição da enzima acetilcolinesterase (AChE). A predição *in silico* das interações de alcaloides dessa fração com a AChE1 de *R. microplus* (*Rm*AChE1) foi realizada por acoplamento molecular. A atividade carrapaticida foi avaliada, utilizando-se os ensaios de imersão de adultos e larvas. Maior efeito sobre parâmetros reprodutivos de teleóginas foi verificado para a FA, com valor de Concentração Efetiva 50% (CE_{50}) (31.6 mg/mL), quatro vezes menor do que o valor do EM (121 mg/mL). A FA induziu mortalidade de larvas (Concentração Letal de 50% - CL_{50} = 13,8 mg/mL). A inibição da atividade da AChE de larvas do carrapato foi observada para EM e FA, sendo a FA mais ativa (Concentração Inibitória 50%- CI_{50} de 0,041mg/mL). As análises químicas da FA permitiram a identificação dos alcaloides prosopina (**1**), juliprosinina (**2**) e juliprosopina

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(**3**). No ensaio *in silico,* observou-se que esses alcaloides podem interagir com o sítio ativo da *Rm*AChE1, principalmente por ligações de hidrogênio e interações cátion-pi. Os alcaloides de *P. juliflora* têm atividade carrapaticida contra *R. microplus*, atuando através do mecanismo anticolinesterásico.

Palavras-chave: Fabaceae, alcaloides, carrapaticida, anticolinesterase, acoplamento molecular.

Introduction

Parasitic diseases impair livestock health and can cause high mortality rate in cattle herds if parasitism rates are high. *Rhipicephalus microplus* is a constant threat to cattle because of the direct and indirect damage that it causes to animal health, thereby leading to diminished reproductive efficiency and milk and meat production (Hue et al., 2015). It is a hematophagous parasite that transmits diseases by acting as a vector for pathogens such as *Babesia bovis* and *Anaplasma marginale* (Adenubi et al., 2016). Use of synthetic acaricides is the most common strategy for tick control; however, increasing resistance to these acaricides has encouraged a search for new bioactive molecules from plants, as treatment alternatives (Rosado-Aguilar et al., 2017).

Prosopis juliflora, popularly known as "algaroba" and "algarobeira", is a shrub belonging to the Fabaceae family that is native to arid and semi-arid regions. This species was introduced into northeastern Brazil more than 50 years ago and is used as a food source for humans and animals because of its high production of pods and the high palatability and nutritional value of the pods (Pegado et al., 2006). Several types of biological activity have been reported for this plant, such as anthelmintic (Lima et al., 2017), insecticidal (Dhivya et al., 2018) and antibacterial (Odhiambo et al., 2015). These effects have been attributed to the alkaloids that are present in this species. The alkaloids of greatest pharmacological importance are juliprosopine and juliprosine (Silva et al., 2013).

The *in vitro* anticholinesterase activity of juliprosopine upon electric eels (*Electrophorus electricus*) acetylcholinesterase enzyme (AChE) was described by Choudhary et al. (2005). AChE is an essential enzyme in the nervous system of ticks and is the main target for organophosphate and carbamate pesticides (Zhou & Xia, 2009).

Because of the scarcity of information on the acaricide activity of *P. juliflora*, the aim of the present study was to evaluate the *in vitro* activity of the methanolic extract (ME) and alkaloid-rich fraction (AF) of this plant on the reproductive parameters of *Rhipicephalus microplus*, and to correlate this effect with inhibition of the AChE. Furthermore, *in silico* assays were performed to characterize the 3D structure of this tick's AChE1 and to predict the possible interaction mode of the major alkaloids of the AF at the active site of the AChE1.

Materials and Methods

Plant material

Pods from *P. juliflora* were collected in the municipality of Senhor do Bonfim, state of Bahia, Brazil, in September 2013. A voucher specimen was deposited at the Botanic Laboratory of Dr. Antônio Nonato Marques, Empresa Baiana de Desenvolvimento Agrícola S.A. (EBDA), Salvador, Bahia (number 5465).

Obtainment of the methanol extract and alkaloid-rich fraction

Air-dried and powdered pods (22.6 kg) from *P. juliflora* were macerated with 9 liters (L) of *n*-hexane for two days. After filtration, the solvent was evaporated under reduced pressure and the remaining plant material was subsequently extracted with methanol (MeOH) (9 L), using the same procedure. The yield of methanol extract (ME) was 0.19%. The ME was concentrated and dried under reduced pressure using a vacuum rotary evaporator (Buchi Rotavapour R-200, Switzerland), which was used to furnish the alkaloid-rich fraction (AF). This was obtained by means of acidic/basic modified extraction as described by Ott-Longoni et al. (1980). The yield of AF was 0.043%.

LC-MS/MS analysis on the alkaloid-rich fraction

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed, coupled with an Esquire 3000^{plus} ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) that was equipped with a CBM-20A controller, LC-20AD pump, SIL-20AC auto-sampler and SPD-20A detector, with an HPLC system connected to this. Separations were performed in a Phenomenex Luna C-18 column (250 × 4.6 mm), with 5-µm particle size. The elution gradient was run using a binary solvent system consisting of water containing 0.05% phosphoric acid (solvent A) and methanol

(solvent B) at a constant flow rate of 1.0 mL/min. The gradient was as follows: 0 min, 0% B; 25 min, 100% B; 35 min, 100% B; 36 min, 20% B; 45 min, 20% B. The injection volume was 20 µL. Data were acquired using a UV detector at 280 nm and 360 nm. The capillary temperature was maintained at 300 °C and the electrospray capillary voltage at 4.5 kV. The LC/MS was performed in positive ionization mode and with the full scan (m/z 100-1,500).

In vitro studies

Rhipicephalus microplus samples

R. microplus from POA strain (Porto Alegre strain) that is sensitive to acaricides currently on the market, and which were free of pathogens such as *Babesia* spp. and *Anaplasma* spp., was obtained from the *Laboratório de Imunologia Aplicada à Sanidade Animal, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul* (UFRGS). This colony has been maintained experimentally through feeding on Hereford calves (*Bos taurus taurus*) since 1976. Calves were purchased from an area that is naturally tick-free (Santa Vitória do Palmar, RS, Brazil; 33°32'2" S, 53°20'59" W) and were maintained in an isolated stall at the Federal Rural University of Rio Grande do Sul (UFRGS), Brazil, to avoid infestation by other ticks. These calves were handled in accordance with the institutional guidelines, as approved by the local ethics committee for animal use (Ethics Committee for Animal Experimentation of the *Universidade Federal do Rio Grande do Sul*).

Engorged females were carefully collected from the cattle, placed in Petri dishes with enough aeration and transported to the Laboratory of Toxicology, School of Veterinary Medicine and Animal Sciences, Federal University of Bahia, Salvador, BA, Brazil. These parasites were selected according to their integrity and motility. Following this, they were washed in distilled water, dried on absorbent paper and separated into two groups. In the first group, engorged females were used to evaluate the acaricidal activity up to 24 h after collection. In the second group, the females were incubated for two weeks (26 ± 2 °C and relative humidity > 80%), to yield larvae that were used to assess the anticholinesterase activity.

Adult Immersion Test (AIT)

The adult immersion test was conducted as described by Drummond et al. (1973). The ticks were weighed and separated into homogeneous groups with 10 females each, according to their weight (1.5 to 2 g). The engorged females were immersed for 5 min in 5 mL of the following treatments: ME (91, 127.6, 178.6, 250 and 350 mg/mL) and AF (16.9, 33.2, 65.1, 127.6 and 250 mg/mL) from *P. juliflora*; negative control (ethanol 70%); and positive control (Diazinon, 2.5 mg/mL), diluted as recommended by the manufacturer (*Agener União Saúde Animal*®). Following the immersion, these ticks were dried on absorbent paper, placed in Petri dishes and incubated for 15 days (26 ± 2 °C and relative humidity > 80%), in order to evaluate oviposition. After this period, the eggs were weighed, transferred to glass tubes and incubated under the same conditions as described above. After 21 days, the larval hatching percentage was estimated visually using a stereomicroscope. Four repetitions were used for each of the treatments.

The data obtained were recorded and used to calculate the following parameters:

Egg production index (EPI) = (weight of eggs/weight of engorged females (g)) x 100 (Bennett, 1974);

Reproductive efficiency index (REI) = (egg-mass weight (g)/weight of engorged females (g)) x % egg hatching x 20,000 (Drummond et al., 1973);

Efficacy of product (EP) = [(REI negative control group - REI treated group)/REI negative control group] x 100 (Drummond et al., 1973).

Larval Immersion Test (LIT)

The LIT was used to evaluate the effect of the AF of *P. juliflora*, most active fraction in AIT, against *R. microplus* larvae (Silva et al., 2009), at concentrations of 4.2 to 65.1 mg/mL. Approximately 100 larvae of 14 to 21 days of age were used, obtained through oviposition from untreated engorged female ticks and collected from naturally infested cattle (Catu, state of Bahia, Brazil). The larvae were put into 5-mL syringes, which were cut next to the needle. The syringe was closed using a fine-weft fabric fixed with an orthodontic rubber band, and the larvae were immersed in the treatments for five minutes and maintained at 27 ± 1 °C and 80 ± 5% relative humidity. An orifice of approximately 0.1 mm in diameter was made in the middle of the syringe. This procedure was repeated for

each concentration of the AF, and for the positive control (Fipronil, 10mg/mL – diluted as recommended by the manufacturer) and negative control (70% ethanol). Larval mortality was recorded after 24 h. Only larvae that had the ability to walk were considered alive. All treatments were set up as three replicates for each concentration tested. Living and dead larvae were counted, and the percentage mortality was calculated as:

$$\%\ \textbf{mortality} = \left(number\ of\ dead\ larvae / total\ number\ of\ larvae\right) x\ 100 \quad (1)$$

In vitro anticholinesterase activity of larvae from *R. microplus*

The anticholinesterase activity of the ME and AF was determined spectrophotometrically, in accordance with the methodology described by Ellman et al. (1961), as modified by Wright & Ahrens (1988). Samples of *R. microplus* larvae (100 mg) were macerated in deionized water (3 mL) and were centrifuged at 1,000 × g for 5 min. Then, the supernatants were used as the enzyme source. In a microtube, 50 µL of ME (final concentration: 0.26, 0.64, 1.6, 4 and 10 mg/mL) and AF (final concentration: 0.001, 0.005, 0.025, 0.125 and 0.625 mg/mL) of *P. juliflora*, the negative control (70% ethanol) and positive control (Eserine/Sigma-aldrich®; final concentration: 0.014 mg/mL) were preincubated with AChE solution (200 µL) during 20 min. at 4 °C. For determination of AChE activity, phosphate buffer solution with pH 8.0 (0.1 M, 2,8 mL) and enzyme solution (200 µL) were homogenized and incubated at 35 °C for 2 min. Then, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB 10 mM, 200 µL) and acetylthiocholine iodide (7.5 mM, 25 µL) were added to initiate the reaction. The absorbance was measured on the spectrophotometer (UV-Vis SP-220 Spectrophotometer, Biospectro, Japan) at 412 nm (0 and 6 min). Each sample was assayed with nine replicates. AChE percentage inhibition was calculated by using the equation:

$$\textbf{Inhibition}\ (\%) = \left[1 - \left(reaction\ rate\ sample / reaction\ rate\ negative\ control\right)\right] \times 100 \quad (2)$$

In silico studies

Comparative modeling of AChE1 from *R. microplus* (*Rm*AChE1)

The amino acid sequence of the cholinergic domain (residues 51 to 528) of *Rm*AChE1 (access code: A0A0F2P2D6), which is available on the UniProt server (UniProt, 2019), was aligned with the primary sequences from the crystallographic structure of the human AChE (*Hs*AChE; PDB ID: 4EY7) and the *Torpedo californica* AChE (PDB ID: 2WFZ), using the Clustal Omega server (EMBL-EBI, 2019a). Both the human and the *Torpedo californica* AChE are available from the Protein Data Bank (RCSB PDB, 2019). From the alignment, 100 models of the *Rm*AChE1 were built using the Modeler 9.18 software (Šali & Blundell, 1993). These were refined through four simulated annealing cycles (flags: autosched.slow; 500 interactions; refine.slow) and were classified according to the scoring function QMEAN6 (SIB, 2019) and the *SSAgree* values (Benkert et al., 2009). Next, the compatibility between the atomic model (3D) of the 10 best models, according to QMEAN score, and its own amino acid sequence (1D) was calculated using the Verify3D server (http://services.mbi.ucla.edu/Verify_3D/, Lüthy et al., 1992). An overall folding quality scale was used for each model. The stereochemistry quality was estimated using the Ramachandran plot (Ramachandran et al., 1963), which was calculated on the PDBsum server (EMBL-EBI, 2019b; Laskowski et al., 1997). The model with the best folding and stereochemistry parameters was used for the molecular docking assays.

Preparation of the *Rm*AChE1 receptor structures and alkaloids

The 3D structure model of the *Rm*AChE1 was prepared for docking assays using the Biopolymer module, which is available on the Sybyl®-X 2.1.1 platform (TRIPOS Associates, 2013). Firstly, hydrogen atoms were added and optimized to prioritize H-bond interactions. Histidine, glutamate and aspartate residues were manually checked for orientation, protonation and tautomeric states. The protonation state of residues was determined using Propka 3.1 (Sondergaard et al., 2011; Rostkowski et al., 2011), with pH = 8.0. Following this, AMBER Force Field 99 charges (Wang et al., 2000) were assigned to the protein. The same protocol was performed for the human isoform (*Hs*AChE; 4EY7).

Major alkaloid structures from the AF (Figures 1 and 2), as well as donepezil, were sketched on the Marvin® Sketch 16.8.29 software (Marvin Sketch 16.8.29, 2017, ChemAxon, 2019) and were later converted into the 3D

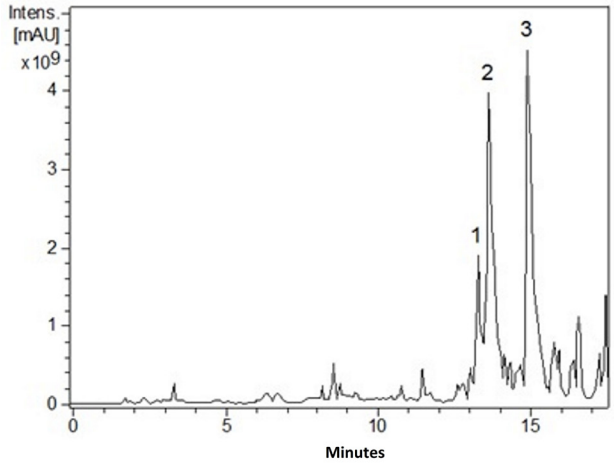

Figure 1. LC-MS/MS chromatogram (positive mode) of the AF of *Prosopis juliflora* (algaroba). Prosopine (1), juliprosinine (2) and juliprosopine (3). Units are in milli Absorbance Units (mAU).

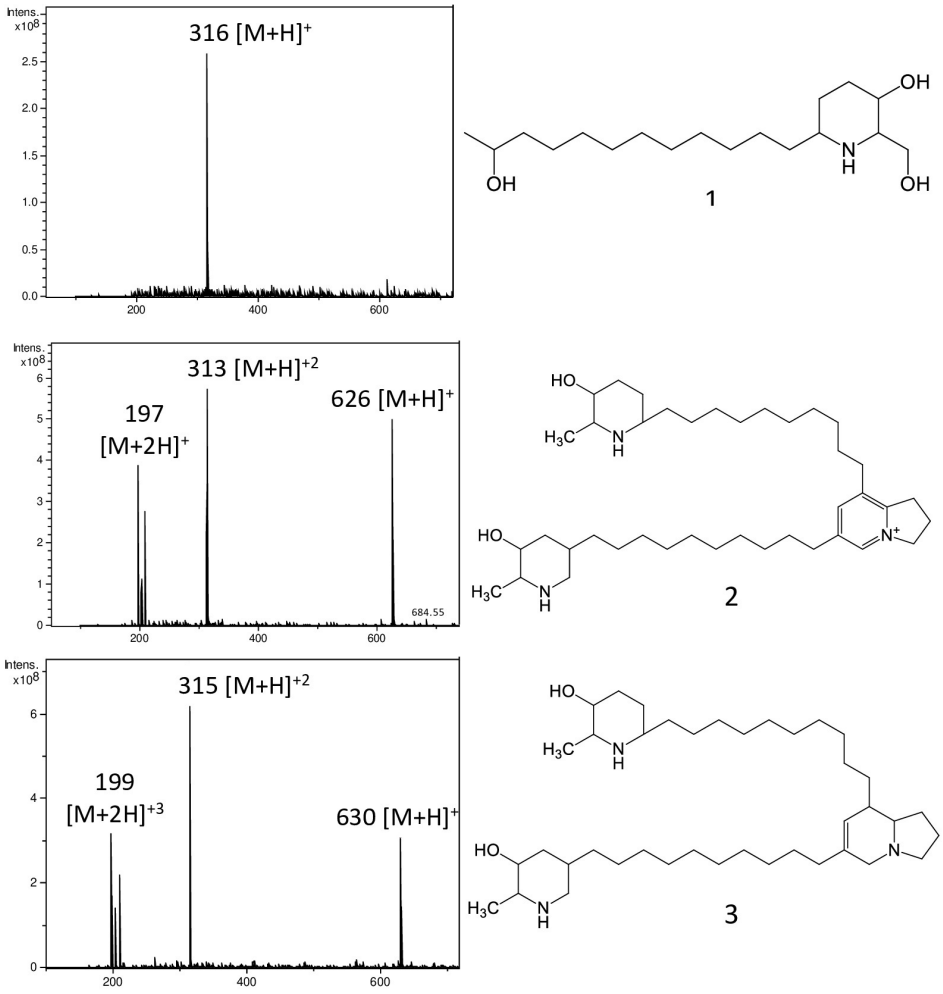

Figure 2. LC-MS/MS spectrum and chemical structures: prosopine (1), juliprosinine (2) and juliprosopine (3).

format using the Concord module (standard parameters), which is available on Sybyl®-X 2.1.1 (TRIPOS Associates, 2013). Afterwards, atomic Gasteiger-Huckel charges (Gasteiger & Marsili, 1980; Hou et al., 2013) were added and the structures were energy-minimized using the conjugate gradient method until the convergence criteria (0.001 kcal/mol or maximum number of interactions = 50,000) were reached at the water dielectric constant (ε = 80.4), in the Tripos force-field (Clark et al., 1989).

Molecular docking studies on piperidine alkaloids

Piperidine alkaloid (ligand) docking was carried out in the GOLD suite v. 5.5 (CCDC, Cambridge, UK). The search space was defined unrestrictedly as a radius of 18 Å, centered on the oxygen (gamma) of the catalytic residue Serine 222. The protein residues remained rigid throughout the calculation, while the ligands were kept flexible (additional N-pyramidal and ring-corner movements were allowed).

Docking simulations were performed using the Lamarckian genetic algorithm (Morris et al., 1998), which is a hybrid of a genetic algorithm and a local search algorithm that is available through GOLD 5.5 (Greenblatt et al., 2007; Jones et al., 1995). The docking parameters were set to default, except for the following: trials of 100 LGA runs for each ligand, initial population size of 250 individuals, random starting position and conformation and 2.5 x 10^6 generations at a selective pressure of 1:1, undergoing mutation (95%), crossover (95%) and migration (10%) in 10 islands and 5 niches. Each docking simulation produced 100 different docked conformations that were later ranked using the Piecewise Linear Potential function (ChemPLP), which was implemented in the GOLD suite (Greenblatt et al., 2007; Jones et al., 1995). Next, the complexes of *Rm*AChE-alkaloids were visually analyzed using the Pymol 1.3 software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). This procedure was also performed for the donepezil structure, in relation to *Hs*AChE.

Statistical analysis

The results obtained from the biological assays were expressed as the mean ± standard deviation (SD). The normality of data was assessed using the Shapiro-Wilk test. The acaricidal evaluation and anticholinesterase activity also underwent univariate analysis of variance (ANOVA) followed by post-hoc Tukey's honest significant test (Tukey´s HSD test, 5%), using the GraphPad Prism statistical software (version 5.0).

Results

Chemical analysis

The LC-MS/MS chromatogram of the AF in the positive mode is shown in Figure 1. Figure 2 presents the MS spectra and the alkaloid structures characterized in this fraction. Alkaloid 1 (T_R.13.4 min) exhibited a protonated molecular ion at *m/z* 316.27 $[M+H]^+$, which fitted the molecular formula $C_{18}H_{37}O_3N$. The MS/MS fragmentation pattern obtained from this alkaloid showed fragment ions at *m/z* 298.32 $[M-18+H]^+$ and 280.33 $[M-36+H]^+$, which were formed by the loss of one and two molecules of water, respectively. Thus, alkaloid **1** was assigned to a prosopine. Alkaloids **2** (T_R.13.7 min) and **3** (T_R.15.0 min) showed protonated molecular ions at *m/z* 626.54 $[M+H]^+$ and 630.62 $[M+H]^+$, which were identified as juliprosinine ($C_{40}H_{72}N_3O_2$) and juliprosopine ($C_{40}H_{76}N_3O_2$), respectively.

Adult immersion test

Table 1 showed the effect of the ME and AF of *P. juliflora* on the reproductive parameters of adult females of *R. microplus*. The ME and AF induced significant reductions ($p < 0.05$) in the oviposition and hatching rates of the ticks, in a concentration-dependent manner. The efficacy of the AF (65.1 to 250 mg/mL) and ME (350 mg/mL) did not differ from that of the positive control (Diazinon; 2.5 mg/mL) ($p > 0.05$). The EC_{50} values were 31.6 and 121 mg/mL for the AF and ME, respectively.

Larval immersion test

Table 2 presents the effect of the AF against the *R. microplus* larvae. The AF had larvicidal activity against *R. microplus*, with LC_{50} value of 13.8 mg/mL. The AF, at the highest concentration tested (65.1 mg/mL), showed a percentage of larvae mortality of 90%.

In vitro anticholinesterase activity against larvae of *R. microplus*

At the highest concentration (0.625 mg/mL), the AF inhibited AChE activity in 95% and did not differ from the positive control (eserine, 0.014 mg/mL) ($p > 0.05$). The IC_{50} of the AF was 0.041 mg/mL. For the ME (10 mg/mL), the maximum percentage enzyme inhibition was 52% (Table 3).

Table 1. Percentage oviposition, hatching and efficacy (mean ± standard deviation) among adult females of *Rhipicephalus microplus*, exposed to the immersion test with the methanolic extract (ME) and alkaloid-rich fraction (AF) of *Prosopis juliflora*. Different letters in columns indicate statistically significant difference (one-way ANOVA followed by the Tukey's HSD test, $p < 0.05$).

Treatments	Concentration (mg/mL)	Egg production (%)	Hatching (%)	Efficacy (%)	EC_{50}
ME	91	$29^{e} \pm 4.2$	$93^{bf} \pm 2.9$	$24^{b} \pm 15.7$	121mg/mL R^2: 0.94
	127.6	$25^{d} \pm 3.5$	$70^{ad} \pm 16.8$	$54^{c} \pm 11.4$	
	178.6	$16^{c} \pm 4.7$	$72^{af} \pm 13.0$	$70^{cf} \pm 7.1$	
	250	$3^{b} \pm 0.3$	$53^{ad} \pm 37.9$	$88^{d} \pm 9.2$	
	350	$9^{a} \pm 7.1$	$45^{ad} \pm 32.2$	$97^{d} \pm 4.2$	
AF	16.9	$41^{f} \pm 3.8$	$42^{de} \pm 7.6$	$45^{e} \pm 4.7$	31.6mg/mL R^2: 0.94
	33.2	$10^{a} \pm 0.4$	$23^{ce} \pm 3.5$	$73^{f} \pm 4.6$	
	65.1	$2^{b} \pm 3.5$	$3^{c} \pm 5.9$	$96^{d} \pm 7.3$	
	127.6	$2^{b} \pm 3.2$	$0^{c} \pm 0.0$	$99^{d} \pm 2.7$	
	250	$0^{b} \pm 0.0$	$0^{c} \pm 0.0$	$100^{d} \pm 0.0$	
EtOH 70%	-	$48^{g} \pm 2.8$	$95^{b} \pm 4.4$	$0.0^{a} \pm 0.0$	-
Diazinon	2.5	$0.0^{b} \pm 0.0$	$8^{c} \pm 15.0$	$98^{d} \pm 2.1$	-

Table 2. Percentage mortality among *Rhipicephalus microplus* larvae exposed to the alkaloid-rich fraction (AF) of *P. juliflora*, positive control (fipronil, 10 mg/mL) and negative control (ethanol 70%). Different letters in columns indicate statistically significant difference (one-way ANOVA followed by the Tukey's HSD test, $p < 0.05$).

Treatments	Concentration (mg/mL)	Mortality (%)	LC_{50}
AF	4.2	$24^{b} \pm 1.4$	13.8 mg/mL R^2: 0,92
	8.5	$25^{b} \pm 2.8$	
	16.9	$50^{c} \pm 6.9$	
	33.2	$64^{d} \pm 6.1$	
	65.1	$90^{e} \pm 1.3$	
EtOH 70%	-	$0^{a} \pm 0$	-
Fipronil	10	$100^{f} \pm 0$	-

Table 3. Percentage inhibition (mean ± standard deviation) of *in vitro* AChE activity of larvae of *R. microplus* exposed to the methanolic extract (ME) and alkaloid-rich fraction (AF) of *P. juliflora*. Positive control: eserine (0.014 mg/mL); negative control: ethanol 70%. Different letters in columns indicate statistically significant difference (one-way ANOVA followed by the Tukey's HSD test, $p < 0.05$).

Treatments	Concentration (mg/mL)	Inhibition AChE activity (%)	IC_{50}
ME	0.26	$19^{c} \pm 1.5$	-
	0.64	$31^{d} \pm 3.1$	
	1.6	$39^{e} \pm 4.2$	
	4	$46^{f} \pm 2.7$	
	10	$52^{g} \pm 1.6$	
AF	0.001	$6^{ab} \pm 1.4$	0.041 mg/mL R^2: 0.99
	0.005	$13^{b} \pm 2.6$	
	0.025	$38^{de} \pm 5.5$	
	0.125	$79^{h} \pm 5.4$	
	0.625	$95^{i} \pm 0.7$	
EtOH 70%	-	$2^{a} \pm 0.5$	-
Eserine	0.014	$100^{i} \pm 0.0$	-

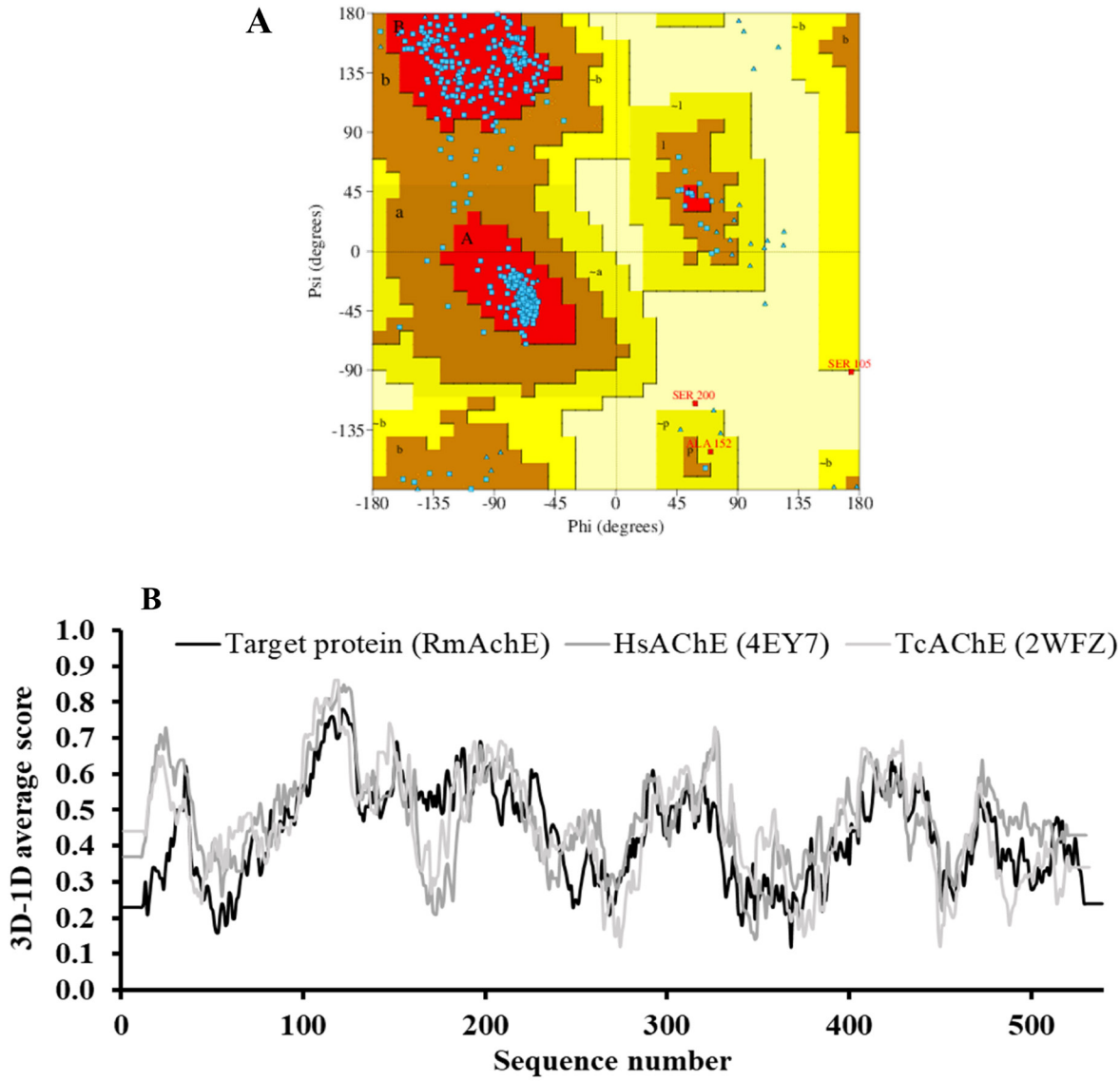

Figure 3. Stereochemical evaluation of the *Rm*AChE1 model. (A) Ramachandran plot: favorable regions (red); additionally allowed (orange); generously permitted (yellow) and not allowed (white); (B) verify-3D plot for averaged 3D-1D values for *Rm*AChE (better model) and HsAChE (template, PDB ID: 4EY7) and TcAChE (template, PDB ID: 2WFZ). Values higher than 0.2 show compatibility between protein folding (3D) and sequences (1D).

In silico studies

Comparative modeling of *Rm*AChE1

100 models of *Rm*AChE1 were built using the Modeler 9.18 software (Šali & Blundell, 1993), from high-resolution crystallography templates: human AChE (HsAChE, PDB ID: 4EY7) and *Torpedo californica* (TcAChE, PDB ID: 2WFZ). Alignment between the template primary sequence and the target (*Rm*AChE1) showed moderate sequential identity (> 42%) (Figure A.1 - Supplementary Material[1]), which enabled proper use of the Modeler software (Webb & Sali, 2016; Šali & Blundell, 1993). After generation of *Rm*AChE1 models, the quality evaluation was performed. The *Rm*AChE1 best-ranked model showed that 98.8% of the residues were in the "allowed region" (Figure 3A) and that 98.7% of the residues presented correct folding (Figure 3B). Hence, it showed stereochemical characteristics,

[1] Methanolic extract (ME), alkaloid-rich fraction (AF), acetylcholinesterase (AChE), adult immersion test (AIT), effective concentration 50% (EC_{50}), liquid chromatography-tandem mass spectrometry (LC-MS/MS), egg production index (EPI), reproductive efficiency index (REI), efficacy of product (EP), acetylthiocholine iodide (ATChI), phosphate buffer (PBS), protein data bank (PDB), human AChE (*Hs*AChE), *Torpedo californica* AChE (*Tc*AChE), *Rhipicephalus microplus* AChE (*Rm*AChE), peripherical anionic site (PAS). Note: Supplementary data associated with this article.

(Figure B.1 - Supplementary Material) and folding that were compatible with the experimental model proteins (PDB ID templates: 4EY7 and 2WFZ). This therefore makes it possible to use this model in future docking studies.

Molecular docking assays on piperidine alkaloids

Docking studies usually require previous steps to evaluate the parameters that are to be used in the search (Cole et al., 2005; Jain, 2008). Briefly, a ligand co-crystallized with a protein is redocked with its receptor. Thus, the deviation values (root mean square deviation, RMSD) of the best ligand posed in relation to co-crystallized coordinates ought to be less than 2 Å (depending on the ligand size). Based on this hypothesis, donepezil was redocked to the receptor HsAChE (PDB ID: 4EY7) (Figure C.1 - Supplementary Material) and the best-ranked pose showed an interaction profile similar to that observed for the co-crystallized structure (RMSD = 1.97 Å). The parameters used for the docking are enough for generation of reliable binding positions with HsAChE and these parameters were applied for *Rm*AChE1 docking studies. Following this, docking of three alkaloids (juliprosinine, juliprosopine and prosopine) was performed at *Rm*AChE1 (Figure 4).

Our docking experiments showed that the 2-methyl-3-piperidinol of juliprosinine (2) perform hydrogen bonding interactions with serine 222, histidine 460 (catalytic site residues) (Figure 4A) and juliprosopine (3) (Figure 4B) hydrogen bonds with glycine 141, serine 222, serine 303 and glycine 305. Those of 2-(hydroxymethyl)-3-piperidinol of prosopine (Figure 4C) with the catalytic site residues (serine 222 and histidine 460) and peripherical anionic site (PAS; tyrosine 144) were also present, along with π-cation interaction with tryptophan 103 (anionic subsite). Π-cation interactions were also observed between the tyrosine 144 residue of the *Rm*AChE1 and the 2,3-dihydro-1H-indolizine (Figure 4A) or hexahydroindolizine (Figure 4B) substructures. Additionally, hydrogen bonds with the PAS residues were assessed between the radical 2-methyl-3-piperidinol of juliprosinine (**2**) and the residues of serine 303 and glycine 305 and between the terminal hydroxyl of prosopine (**1**) and glutamate 300.

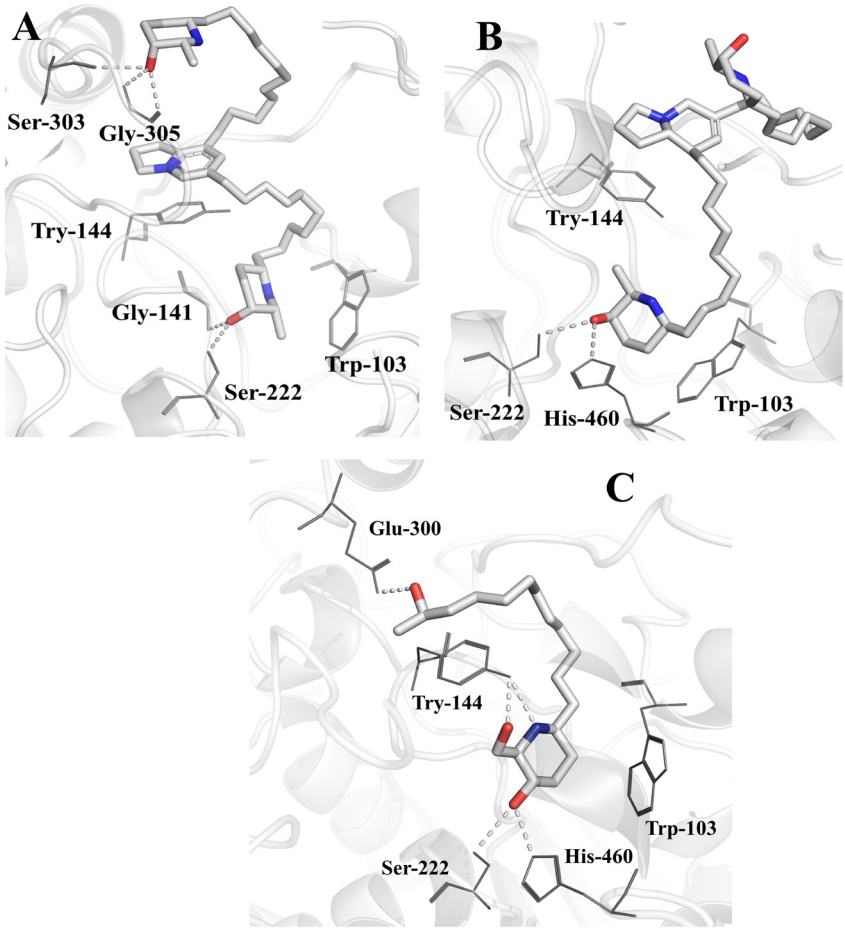

Figure 4. Main interactions between the *Rm*AChE1 residues and piperidine alkaloids: (A) juliprosinine; (B) juliprosopine; and (C) prosopine. These images were generated using the Pymol 1.3 software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Discussion

In the present study, the ME and AF of *P. juliflora* were shown to have acaricidal activity against *R. microplus*. The AF was four times more effective on adult females of *R. microplus* than was the ME, thus suggesting that the alkaloids present in this plant are the compounds responsible for this activity. In addition, the AF was more active on larvae (LC_{50}=13.8 mg/mL) than on adult females (EC_{50}=31.6 mg/mL) of *R. microplus*. These differences may be related to the greater mass of individual engorged adults and thinner cuticle of *R. microplus* larvae, which would make these stages more sensitive than the adult females (Cruz et al., 2016; Conceição et al., 2017). The LC-MS/MS analyses on the AF led to identification of three alkaloids (prosopine, juliprosinine and juliprosopine). These compounds were characterized through comparisons with data in the literature (Ott-Longoni et al., 1980; Ahmad et al., 1989; Singh & Swapnil, 2011; Singh & Verma, 2012; Santos et al., 2013). Other types of antiparasitic activity exhibited by this plant, such as insecticidal (Yadav et al., 2015; Dhivya et al., 2018) and anthelmintic (Lima et al., 2017), have also been described.

These findings are the first scientific reports on the anti-tick activity of the alkaloids of *P. juliflora*. The activity of alkaloid and non-alkaloid fractions of *Leucas indica* against *Rhipicephalus (Boophilus) annulatus* was studied previously, and only the alkaloid fraction (50 mg/mL) induced adult tick mortality (66.67%) and inhibition of fecundity (55.16%) (Divya et al., 2014).

From our evaluation of the anticholinesterase effect, the inhibition of AChE activity produced by the ME and AF of *P. juliflora* makes it possible to include them in the group of potent inhibitors (> 50% inhibition) of this enzyme, according to the classification of Vinutha et al. (2007). The AF was more active at a lower concentration (IC_{50} = 0.041 mg/mL) than the ME (10 mg/mL; 52%), thus indicating that alkaloids are the bioactive compounds of *P. juliflora* responsible for the anticholinesterase activity. Previous studies reported that juliprosopine, isolated from *P. juliflora,* was active *in vitro* against the AChE of electric eels (*Electrophorus electricus*) (Choudhary et al., 2005).

Our work indicates that the alkaloids of *P. juliflora* have the same macromolecular target as do organophosphate and carbamate pesticides. According to Tan et al. (2011), inhibition of the AChE leads to increased levels of the neurotransmitter acetylcholine and to paralysis and death of the tick.

With the aim of understanding the interactions of these alkaloids (presented in the AF) with the AChE of *R. microplus* (*Rm*AChE), *in silico* studies were performed on *Rm*AChE1. The higher affinity with the substrate (acetylcholine; ACh) and higher conversion rates of *Rm*AChE2 and *Rm*AChE3 that were previously observed (Temeyer et al., 2010) suggest that *Rm*AChE1 is very important for tick survival. However, the lack of data on the crystallographic structure of *Rm*AChE limits both the designing of new acaricidal compounds and knowledge of the mechanism of action of these molecules (Williams et al., 2005; Lionta et al., 2014; Ferreira et al., 2018). Thus, comparative modeling (homology modeling) plays a significant role in reducing these limitations because it enables investigation, at the atomic level, of the molecular interactions of anticholinesterase compounds through molecular docking (Schmidt et al., 2014).

Since these *in vitro* assays with these compounds do not allow assessment of the molecular inhibition mechanism against *Rm*AChE, docking studies were conducted. These were based on previous results relating to the non-competitive inhibition mechanism of juliprosopine against the AChE of *E. electricus* (Choudhary et al., 2005). Docking assays for other two alkaloids were conducted in relation to *Rm*AChE1 because high Tanimoto coefficients (Bajusz et al., 2015; Rogers & Tanimoto, 1960) were achieved (juliprosinine T.C. > 95% and prosopine T.C. = 45%). This coefficient describes the percentage of structural similarity between two distinct compounds (Bajusz et al., 2015): the more similar their structures are, the higher the Tanimoto coefficient is.

The docking showed that the alkaloids seem to interact preferentially with the residues of the catalytic site, anionic subsite and PAS of *Rm*AChE1 (Figure 4). Although the binding mode was distinct from that proposed before (Choudhary et al., 2005), the patterns of interactions were very similar (Figure 4). Our docking results corroborated what had been described for other known ligands of AChEs, such as tanshinone (Cheung et al., 2013), galantamine (Greenblatt et al., 2007), huperzine A and donepezil (Cheung et al., 2012). For instance, the residue tryptophan 286 (present in PAS of HsAChE) has an important π-π stacking interaction with donepezil (PDB ID: 4EY7) and tanshinone (PDB ID: 4M0E). However, at *Rm*AChE1, this residue was replaced by threonine (Thr 301), thus leading to the loss of this interaction (Figure 4 and A.1), which reduced the affinity of these compounds against the enzyme (Swale et al., 2013). The docking results suggest that this lack of interaction was compensated by the π-cation interaction between the 2,3-dihydro-1H-indolizine or hexahydroindolizine from alkaloids and tyrosine 144 from *Rm*AChE1 (Figure 4).

π-cation and π-π interactions with tryptophan 86 (anionic site of the HsAChE) and hydrogen bonds with serine 203 also seem to be important for the mechanisms for inhibition caused by huperzine A (PDB ID: 4EY5), galantamine (PDB ID: 1DX6) and donepezil (PDB ID: 4EY7). These interactions were similar to our results between the 2-methyl-3-piperidinol and 2-(hydroxymethyl)-3-piperidinol groups and the tryptophan 103 and serine 222 residues of *Rm*AChE1 (Figure 4).

Conclusion

The alkaloids of *P. juliflora* presented an *in vitro* acaricidal effect on the larvae and engorged females of *R. microplus*, and they inhibited acetylcholinesterase. An *in silico* assay on the main alkaloids obtained from the alkaloid-rich fraction (juliprosopine, juliprosinine and prosopine) suggested that these compounds preferentially interacted at the catalytic and PAS sites of *Rm*AChE1. These interaction profiles are similar to those described for several known AChE inhibitors. To achieve a better description of this mechanism of action, additional in vitro studies on recombinant RmAChE1 and in silico molecular dynamic simulations are required.

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Supplementary Material

Supplementary material accompanies this paper.

Figure A.1 - Sequence alignment between diverse cholinesterase domains: RmAChE 1 (Target protein), HsAChE (Template, PDB ID: 4EY7) and TcAChE (Template, PDB ID: 2WFZ). The identical residues were highlighted in black and residues with similarity greater than 70%, according to the BLOSUM 62 matrix, were highlighted in gray.

Figure B.1 - Comparison of Donepezil conformations at docking studies (black) and crystallographic structure (PDB ID: 4EY7, gray) at HsAChE site.

Figure C.1 - Stereochemical evaluation (Ramachandram plot) of the templates. Left - HsAChE (PDB ID: 4EY7) and right - TcAChE (PDB ID: 2WFZ).

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