

Anonaine from *Annona crassiflora* inhibits glutathione S-transferase and improves cypermethrin activity on *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887)

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ABSTRACT

Rhipicephalus (Boophilus) microplus (Canestrini, 1887) is one of the most important ectoparasites of cattle, causing severe economic losses in tropical and subtropical regions of the world. The selection of resistance to the most commonly used commercial acaricides has stimulated the search for new products for tick control. The identification and development of drugs that inhibit key tick enzymes, such as glutathione S-transferase (GST), is a rational approach that has already been applied to other parasites than ticks. In this context, alkaloids such as anonaine display several biological activities, including an acaricidal effect. This study aimed to assess the specific inhibition of the *R. microplus* GST by anonaine, and analyze the effect on ticks when anonaine is combined with cypermethrin. For this purpose, a molecular docking analysis was performed using an *R. microplus* GST three-dimensional structure model with anonaine and compared with a human GST-anonaine complex. The absorption, distribution, metabolism, excretion, and toxicity properties of anonaine were also predicted. Then, for *in vitro* analyses, anonaine was isolated from *Annona crassiflora* (Martius, 1841) leaves. The inhibition of purified recombinant *R. microplus* GST (rRmGST) by anonaine and the effect of this alkaloid on cypermethrin efficacy towards *R. microplus* were assessed. Anonaine has a higher affinity to the tick enzyme than to the human enzyme *in silico* and has moderate toxicity, being able to inhibit, *in vitro*, rRmGST up to 37.5% in a dose-dependent manner. Although anonaine alone has no activity against *R. microplus*, it increased the cypermethrin effect on larvae, reducing the LC₅₀ from 44 to 22 µg/mL. In conclusion, anonaine is a natural compound that can increase the effect of cypermethrin against *R. microplus*.

1. Introduction

The cattle tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) poses a severe economic threat to livestock producers through physical effects on infested animals and diseases caused by the transmission of parasitic protists (Kumar et al., 2013). It is estimated that *R. microplus* causes annual losses in the Brazilian cattle herd of up to US\$ 3.2 billion (Grisi et al., 2014).

Tick control is usually carried out through the repeated use of chemical acaricides, such as synthetic pyrethroids (Kumar et al., 2013), which has led to increased selection of acaricide resistance among tick populations, in addition to promoting contamination of the environment and food products (Kaewmongkol et al., 2015).

Plants defend themselves against pests by producing several phytochemicals that have been considered potential alternatives for tick control (Guneidy et al., 2014). For instance, anonaine, an alkaloid

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present in the plant *Annona crassiflora* (Martius, 1841) (Annonaceae), a tree native to the Brazilian Cerrado popularly known as “araticum”, is a bioactive compound displaying several biological properties, including antiparasitic activity (Li et al., 2013).

Various enzyme inhibitors have been studied to develop control methods against parasites (Olivares-Illana et al., 2006; Braz et al., 2019; Cuevas-Hernández et al., 2020), based on the identification of molecules that induce selective inhibition of parasite over host enzymes (Ahmad et al., 2008; Moraes et al., 2011; Ozelame et al., 2022). Based on these previous results, the enzyme glutathione S-transferase (GST) can be considered a target for developing antiparasitic drugs. Each of the GST subunits has its active site that is composed of a glutathione (GSH) binding site (G site) and an electrophilic substrate binding site (H site) (Prade et al., 1997). GSTs play an essential role in detoxifying xenobiotics (Mannervik, 1985; Mannervik et al., 1988; Hamza and Dailey, 2012). Compounds capable of inhibiting the tick's GST activity to interrupt its detoxification system, could provide an alternative form of control (Guneidy et al., 2014; Ozelame et al., 2022). As alkaloids are among the natural products capable of inhibiting GST (Mangoyi et al., 2010; Azeez et al., 2012; Divya et al., 2014; Behera and Bhatnagar, 2019), anonaine is a potential candidate for the control of *R. microplus* through the inhibition of this enzyme.

Recently, *in silico* techniques have facilitated the discovery of new drug candidates (Alvarez, 2004; Choubey and Jeyaraman, 2016; Ganesan, 2016; Roche and Bertrand, 2016; Saramago et al., 2018). For instance, through molecular docking, drug candidates can be recognized, and the potential for their optimization can be explored as molecular interactions between ligands and target molecules can be analyzed and modelled (Wadood et al., 2013).

Given the scientific and economic importance of the development of new acaricide products against ticks and considering that GST is a target enzyme essential in the physiology of the ticks, this study used *in silico* and *in vitro* assessments to analyze the potential use of anonaine as a specific tick GST inhibitor. By decreasing the activity of this enzyme one can interfere with the detoxification of cypermethrin, thereby increasing the effectiveness of this synthetic pyrethroid.

2. Methodology

2.1. Construction and validation of the glutathione S-transferase (GST) model

The GST sequence of *R. microplus* (GenBank number AAL99403.1) was used as a query on the Phyre 2 server (Kelley et al., 2015), with normal modelling mode. The created model was then validated using the PROCHECK 3.0 server (Laskowski et al., 1993).

2.2. Anonaine structure and ADMET features

The anonaine structure was obtained from the PubChem database (CID: 160597) in mol2 format and optimized in the Avogadro program (Hanwell et al., 2012). ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of anonaine were analyzed using PreADMET software (Kwang, 2005). The ADMET analyses were carried out according to the specific classifications and parameters (Van De Waterbeemd and Gifford, 2003; Tong et al., 2021).

2.3. Molecular docking of GST from *R. microplus* and human with anonaine

To analyze the potential inhibitory activity of anonaine to the *R. microplus* enzyme, molecular docking was carried out in the H-site of both a human and a tick GST, using Molegro Virtual Docker 6.0 (MVD) software. The structure of the human GST complexed with the inhibitor N11 (6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)sulfanyl]hexan-1-ol) was obtained from the Protein Data Bank (www.rcsb.org) at 1.8 Å resolution

(PDB ID: 3IE3 – chain A).

The human GST structure was employed for re-docking simulations by fitting the N11 to the enzyme using 32 docking protocols. For this purpose, statistical analysis of coupling results and scoring functions (SAnDReS) were used (Xavier et al., 2016). The algorithms were valid if the re-docking results had a root square mean deviation (RMSD) less than 2 Å from the original structure (Yusuf et al., 2008). The re-docking protocol result with the lowest RMSD was selected for molecular docking simulations.

The structures of anonaine and human GST were imported into the MVD workspace in ‘mol2’ format. The GST's structures were prepared (always assigning bonds, bond orders and hybridization, charges and tripos atom types; always creating explicit hydrogens and always detecting flexible torsions in ligands) using the utilities provided in MVD. Molecular docking was carried out inside a virtual docking sphere of 15 Å radius and the following centre coordinates: X: 6.06; Y: 3.61; Z: 28.00 Å. Ten independent runs were conducted, and the results were expressed in MolDock score. The more negative the number, the better the binding (Hall Jr and Ji, 2020). The same parameters were used to perform the molecular docking of anonaine onto the *R. microplus* GST. It is noteworthy that after superimposing the structures of the human and tick GSTs used in this study, an RMSD of 1.1 Å was obtained while their sequences have an amino-acid identity of 28.8%.

The best pose of both GSTs with anonaine was visualized and analyzed using the PyMOL Molecular Graphics System v1.3 program (<http://www.pymol.org/>) and the residues of the GSTs interacting with anonaine were analyzed using Discovery Studio Visualizer software.

The *R. microplus* and human (Linnaeus, 1758) GST sequences were aligned using Clustal Omega software (Sievers et al., 2011), and the residues interacting with anonaine (taken from the docking results with both GSTs) were highlighted in the alignment.

2.4. Extraction and purification of anonaine

The extraction and purification procedure followed a methodology adapted from Chen et al. (2001). Leaves of *Annona crassiflora* were collected at Parque Nacional Chapada das Mesas (07°07'47.1" S, 4°25'36.8" W), Carolina, Maranhão, Brazil, in April 2018. A specimen (Exsiccate number MG 222438) was deposited in the Museu Paraense Emílio Goeldi (MPEG) or Goeldi Museum, located in Belém, Pará, Brazil.

The leaves were dried in a circulating air oven at 50 °C, ground (300 g), and subjected to cold extraction using initially petroleum ether and then methanol (3 × 1 L, each), resulting in 15.54 g of Ethereal Extract and 35.45 g of Methanolic Extract, respectively. The analysis by thin-layer chromatography (TLC), using Dragendorff reagent, indicated the presence of alkaloids in the methanolic extract. Therefore, about 10 g of the methanolic extract was subjected to conventional acid-base treatment, yielding the alkaloid enriched fraction (m: 0.57 g).

A part of the fraction (0.4 g) was subjected to chromatographic fractionation in a silica gel column chromatography previously treated with a 5% NaHCO₃ solution and eluted with gradients of petroleum ether: CH₂Cl₂, then gradients of CH₂Cl₂: EtOAc, and finally gradients of EtOAc: CH₃OH, resulting in 50 fractions of 25 mL each. The obtained fractions were analyzed by TLC in different solvent systems and gathered into 7 groups. Group 3 (40.5 mg) was subjected to TLC using CH₂Cl₂: MeOH (8.0:2.0, v/v) as eluent, and a single spot was found on the plate. The identification of anonaine was done by comparison with standards and analysis of the mass spectrum.

2.5. Expression and purification of glutathione S-transferase of *Rhipicephalus microplus* (rRmGST)

A DNA fragment containing the entire coding sequence of a *R. microplus* GST was cloned in previous studies (Vaz et al., 2004; Ndawula et al., 2019). Then, the recombinant GST (rRmGST) was expressed and purified as previously described (Ndawula et al., 2019).

Briefly, *Escherichia coli* (Migula 1895) BL21(DE3) was transformed with plasmid and the rRmGST expression (in SOB medium) was induced by 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside, Thermo Fisher Scientific, Waltham, MA, USA) for 6 or 18 h at 37 °C. The culture was centrifuged at 16,000×g for 10 min at 4 °C and the pellet was washed with PBS 7.2 and lysed using an ultrasonic homogenizer with 5 cycles of 30 pulses for 30 s (Pulse Sonics Vibra-cell VCX 500–700, Sonics & Materials, Inc., Newtown, CT, USA).

The supernatant was loaded onto an affinity chromatography column of GSTrap 4B (GE Healthcare, Chicago, IL, USA), previously equilibrated with binding buffer (PBS pH 7.4). After being washed with the same buffer, the rRmGST was eluted with 50 mM Tris-HCl pH 8.0 containing 10 mM reduced glutathione (GSH). The expression and purification of rRmGST were monitored by SDS-PAGE and western blotting using anti-rRmGST rabbit serum (Ndawula et al., 2019).

2.6. GST enzymatic activity and inhibition by anonaine

The enzymatic activity of purified recombinant GST was determined using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich, Saint Louis, MO, USA) and 3,4-dichloronitrobenzene (DCNB) (Sigma-Aldrich) at 25 °C with a VersaMax™ Microplate Reader. Readings were performed at 340 nm for 30 min at 15 s intervals, as previously described (Vaz et al., 2004; Habig et al., 1974). Substrates CDBN 3 mM and DCNB 1 mM were diluted in methanol and added to the reaction mixture containing 100 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA, and 3 mM GSH. Tests were performed in 96-well microplates with 10 µL (0.7 µg) of recombinant protein in a total volume of 100 µL. The background activity, which was subtracted from the data, was determined using buffer, GSH, and CDBN, without enzyme.

For the inhibition tests, anonaine was diluted in 1% DMSO at 10 mg/mL (stock solution). The inhibition of GST by anonaine was carried out at concentrations in the range of 0.075–0.5 mg/mL. Inhibition tests were with 10 µL of recombinant protein in 100 µL of total volume. The assay in which anonaine was replaced by PBS represented 100% enzymatic activity. As a negative control, GST, CDBN, GSH, and DMSO (0.1%) were used. The assays were performed in two independent assays, each in duplicate.

2.7. Ticks

Ticks of the Santa Rita strain were collected from naturally infested Girolando cattle on a farm located in the municipality of Santa Rita (03°08'37"S, 44°19'33"W), MA, Brazil, and maintained through artificial infestation on calves at the facilities of the Federal University of Maranhão (UFMA). This study was approved by the Ethics Committee on Animal Experimentation of UFMA, Brazil, under protocol number 23115.004153/2022–58.

2.8. Larval immersion test

The larval immersion test was performed according to Klafke et al. (2006), in triplicate. From the anonaine stock solution (10 mg/mL), solutions at 0.5 and 0.1 mg/mL final concentrations, in 1% ethanol and 0.02% Triton X-100 were tested. Cypermethrin was prepared at 20 mg/mL (stock solution) in 1% ethanol and 0.02% Triton X-100 and tested at 3.0, 1.2, 0.48, 0.19, 0.07, 0.03, 0.0123, 0.004, 0.002 and 0.0008 mg/mL. Cypermethrin was combined with anonaine (same concentrations as described above) in the tests on tick larvae. The control group was treated with a 1% ethanol and 0.02% Triton X-100 solution.

Approximately 500 larvae were immersed for 10 min in a mixture of anonaine and cypermethrin and transferred to a filter paper base. Then, approximately 100 larvae were transferred to a clean filter paper package (8.5 × 7.5 cm) closed with plastic clips. The packets were incubated for 24 h at 27 ± 1 °C with relative humidity ≥80%. Ticks were

observed for 5 min. Dead (no movement) and alive larvae were manually counted. The tests were carried out in triplicate.

2.9. Adult immersion test (AIT)

For the adult immersion test (AIT) (Drummond et al., 1973), anonaine (at 0.5 and 0.1 mg/mL final concentrations) and cypermethrin (3.7 mg/mL final concentration) were prepared as previously described and mixed in a solution. The tests were carried out in triplicate.

Engorged females of *R. microplus* with homogeneous body mass (n = 180) were divided into six groups (n = 10) as follows: 1) Control: 1% ethanol and 0.02% Triton X-100 solution (v/v); 2) 3.7 mg/mL cypermethrin; 3) 3.7 mg/mL cypermethrin and 0.1 mg/mL anonaine; 4) 3.7 mg/mL cypermethrin and 0.5 mg/mL anonaine; 5) 0.1 mg/mL anonaine; 6) 0.5 mg/mL anonaine. The cypermethrin concentration used in AIT was determined by Ghosh et al. (2017). Ticks of each group were immersed in their respective solution for 5 min, washed, and dried on absorbent paper.

The engorged females from each group were incubated at 27 ± 1 °C and RH ≥ 80%, for 15 days. After weighing the collected eggs and incubating them for 25 days at the same temperature and humidity, the percentages of reduction in both oviposition and hatching were assessed (Bennett, 1974; Lopes et al., 2013; Drummond et al., 1973).

2.10. Statistical analysis

For the enzymatic inhibition, larval, and adult immersion tests, all means obtained were statistically analyzed by Analysis of Variance (ANOVA), followed by Tukey's test (p < 0.05). The results were initially transformed to log (X), and the percentage of mortality was normalized; subsequently, non-linear regression was performed to obtain the LC₅₀ (50% lethal concentration) values using GraphPad Prism 8.0.2 software (GraphPad Inc., San Diego, CA, USA). The significance of each concentration in the tests was established when the calculated confidence intervals do not overlap (Roditakis et al., 2005).

3. Results

3.1. Modelling of the three-dimensional (3D) structure of the GST of *R. microplus*

The best template identified to prepare a reliable 3D structure model of *R. microplus* GST (Supplementary Fig. 1) using the Phyre2 web server was a *Gallus gallus* GST (Chain A, PDB:1C72), with 37.21% identity and 98% of coverage. The model dimensions were X: 51,117, Y: 42,329, Z: 55,806 Å, with 100% modelling confidence. The stereochemistry of the refined protein model revealed that of the 220 amino acid residues of the GST of *R. microplus*, 91% were situated in the most favorable region of the Ramachandran plot (Supplementary Fig. 2).

3.2. Re-docking and molecular docking

Re-docking protocol number 23 (Xavier et al., 2016), which uses plants score as score function, and the iterated simplex (Ant Colony Optimization) as search algorithm, resulted in an RMSD of 1.9 Å (docking RMSD value for human GST, PDB: 3IE3, with N11 inhibitor) and was selected for molecular docking simulations in this study.

As a result of the molecular docking simulations, anonaine showed higher affinity to the *R. microplus* GST, with lower binding energy (−91.355) for this enzyme, compared to the binding energy for the human GST (−85.249). The predicted interactions with the amino acids from each of the GSTs (from the best pose for each GST) with anonaine are highlighted in the alignment of the two GST sequences (Fig. 1). Anonaine was found to interact with the amino acids: Thr 10, Thr 11, Ala 12, Tyr 35, Glu 36, Phe 37, Gly 38, Pro 39, Ala 40, Tyr 43, Pro 209, Met 211, Ala 212, Pro 213 of *R. microplus* GST (Fig. 1 and Supplementary

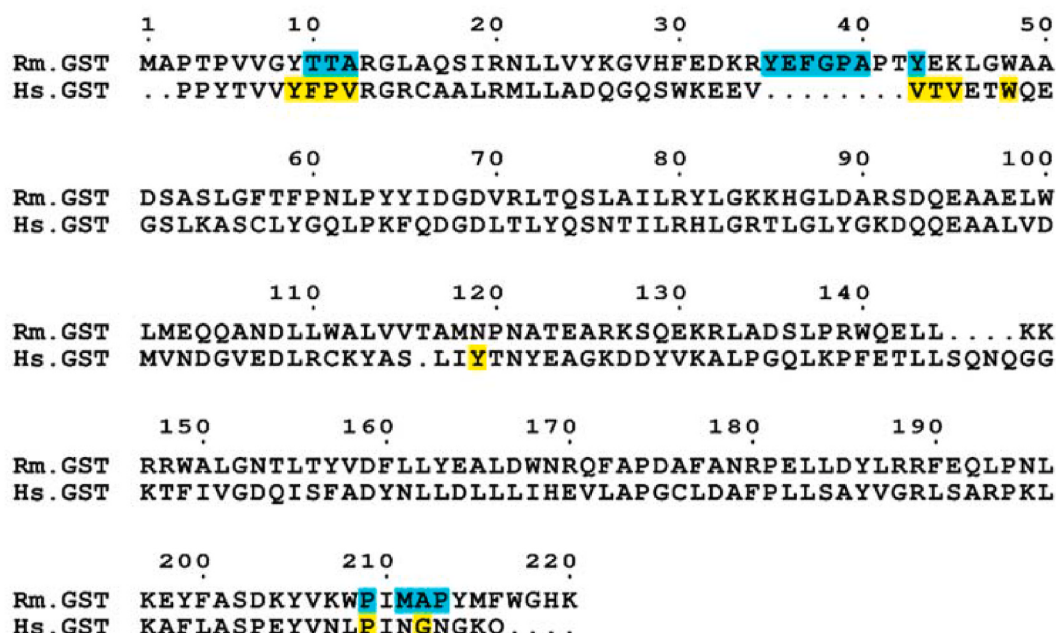


Fig. 1. Protein sequence alignment of the human GST (Hs.GST), (PDB ID: 3IE3-Chain A) and *Rhipicephalus microplus* GST (Rm.GST). Residues of human GST and tick GST interacting with anonaine are highlighted in yellow and blue, respectively.

Table 1).

3.3. ADMET analysis

The predicted ADMET properties of anonaine are shown in [Supplementary Table 2](#). Anonaine is predicted to have good human intestinal absorption (96.493%), medium permeability in the Caco-2 cell model (47.681 nm/seg), low permeability in the Blood-Brain Barrier (BBB) model (0.9849), high permeability in the MDCK cellular system (>25 nm/s), and a high plasma protein binding rate (65.565%). Regarding metabolism, anonaine is predicted to have inhibition ability on CYP2D6

and CYP3A4; to show mutagenic Ames toxicity and a low value of toxicity in the algae test (0.055948 mg/L), suggesting it will have moderate side effects to the mammals.

3.4. Isolation of alkaloid anonaine and rRmGST

Anonaine was isolated from the leaf methanolic extract of *A. crassiflora* as shown in the HPLC analysis (Fig. 2). The positive-mode mass spectrum showed a molecular ion of m/z 266 $[M+H]^+$, with fragments of m/z 249, m/z 219, and m/z 191, indicating the initial loss of the amine group and the CH_2O and CO groups.

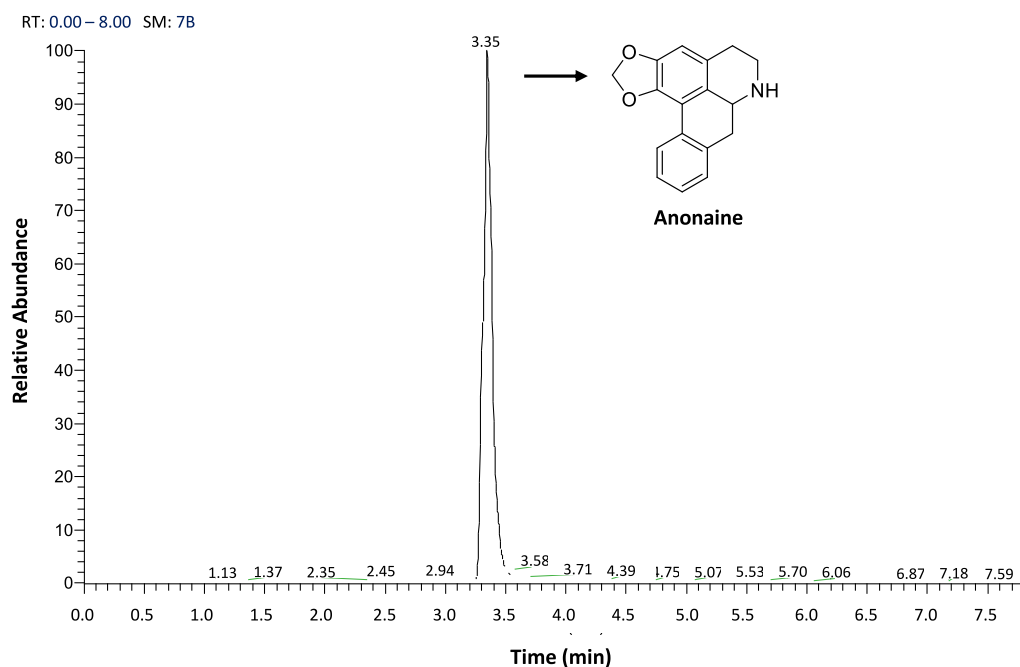


Fig. 2. Chromatogram of total ions of anonaine, isolated from *Annona crassiflora*. Inset: anonaine structure.

A single protein band was observed in SDS-PAGE and Western blot analyses of rRmGST purified by GSH affinity-column chromatography, confirming the enzyme's identity and purity (above 97%) (Fig. 3).

3.5. *In vitro* inhibition of rRmGST by anonaine

The inhibitory activity of anonaine on the rRmGST was determined at fixed concentrations of CDNB (3 mM) and GSH (3 mM). It was observed that *R. microplus* GST was inhibited by anonaine in a concentration-dependent manner (Fig. 4).

3.6. Effect of anonaine and cypermethrin on larvae and adults of *Rhipicephalus microplus*

Addition of anonaine increased the effect of cypermethrin on larvae; at a concentration of 0.5 mg/mL resulting in a reduction in the cypermethrin's LC₅₀ from 44 to 22 µg/mL, although anonaine itself did not show activity toward *R. microplus* larvae at the tested concentrations (Table 1). Anonaine had an effect of $6.24 \pm 8.74\%$ and $14.26 \pm 25.82\%$ in engorged females at 0.1 and 0.5 mg/mL, respectively and did not alter the cypermethrin effect on adults of *R. microplus*.

4. Discussion

The search for alternatives to control *R. microplus* is one of the biggest challenges for cattle production as illustrated by several reports about emergence of multi-resistant tick populations (Tavares et al., 2022). This study presents *in silico* and *in vitro* evidence of inhibition of *R. microplus* GST by the purified plant alkaloid anonaine, which improved the cypermethrin *in vitro* larvicidal effect.

First, the potential of anonaine to inhibit *R. microplus* GST was evaluated *in silico* after the construction and validation of an *R. microplus* GST structure model (Supplementary Fig. 2). The Ramachandran plot of the tick GST modelled structure showed 91% of the residues in the most favorable regions (Supplementary Fig. 2). This result was adequate since a percentage of CORE residues higher than 90% indicates that a model has a good resolution (Laskowski et al., 2013).

To identify the best docking protocol, a re-docking experiment was carried out with the human GST and the N11 inhibitor, and an RMSD of 1.9 Å was obtained. The algorithms are valid if the re-docking results have an RMSD less than 2 Å from the original structure (Hecht and Fogel, 2009). After the tick and human GST structures superimposition, the RMSD obtained was 1.1 Å. The percentage of amino-acid identity between the two protein sequences is a mere 28.8%, but the low RMSD value indicates high structural similarity between the two structures.

Additionally, protocols for molecular docking consider that 3D structures of two protein sequences having an identity higher than 25% are sufficiently similar for comparative docking studies (Shen et al., 2013). Based on these results, the same docking protocol was used for both GST structures in this study. According to the molecular docking results, anonaine would have a higher affinity for *R. microplus* GST than for human GST (Fig. 1 and Supplementary Fig. 1).

The residues of the human GST interacting with anonaine were not the same as the *R. microplus* GST interacting residues (Supplementary Fig. 1 and Supplementary Table 1), suggesting a different mode of ligation between anonaine with the parasite and with the mammalian enzymes. This could thus be helpful for the development of selective drugs (Ahmad et al., 2008; Moraes et al., 2011).

The predicted ADMET properties of anonaine with different parameters analyzed by the PreADMET tool shown in Supplementary Table 2, suggest that anonaine has moderate toxicity and no carcinogenic potential. All values obtained in the results with anonaine were compared to standard values reported in the literature (Ames et al., 1972; Yee, 1997; Van De Waterbeemd and Gifford, 2003; Alliance, 2016; Wadapurkar et al., 2018; Ferreira et al., 2020; Pereira and Bruno, 2021; Tong et al., 2021). Also, it is suggested that natural alkaloid anonaine is less toxic to mammals than cypermethrin. However, additional studies to elucidate anonaine's mechanism of action, pharmacology, toxicity, and pharmacokinetics are necessary to explore possibilities for its optimization and veterinary application of derived products.

Alkaloids exhibit multiple biological activities, and there are already several drugs commercially available derived from natural plant alkaloids (Debnath et al., 2018). In this study, anonaine was isolated from leaves of *Annona crassiflora* in an amount and quality adequate to perform the immersion tests (Fig. 2).

The inhibition of rRmGST activity increased with the increase in the anonaine concentration (Fig. 4), revealing the capacity of an alkaloid to inhibit tick GST. A similar result has been reported for alkaloids isolated from the plant *Rauvolfia tetraphylla* (Linnaeus, 1753) that inhibited the GST activity of *Setaria cervi* up to 64% at 1 mg/mL (Behera and Bhatnagar, 2019).

The most important finding was that the combination of anonaine (0.5 mg/mL) with cypermethrin increased the toxicity of the pyrethroid 2-fold against *R. microplus* larvae (Table 1). Plant alkaloids have been demonstrated to possess acaricidal activity against *R. microplus* and *R. annulatus* (Divya et al., 2014; Silva et al., 2021). For instance, alkaloids and glycosides detected in a *Datura metel* extract had synergistically inhibitory effects against *R. microplus* engorged females (Ghosh et al., 2015). Moreover, an alkaloid-rich fraction from *Prosopis juliflora* (Sargent 1902) was responsible for activity against adult females of *R. microplus* (Lima et al., 2020). In addition, this alkaloid-rich fraction was more active on larvae than on adults. However, many approaches, including chemical and formulation modifications can be utilized to improve drug properties and increase the biological effect against adult ticks. The different susceptibility between *R. microplus* larvae and adults for the alkaloids may be explained since larvae have a thinner cuticle than adults (Conceicao et al., 2017; Cruz et al., 2016). In this study, anonaine alone was ineffective against *R. microplus* larvae. Our result suggests that this alkaloid, by inhibiting the *R. microplus* GST, interferes negatively with the cypermethrin detoxification system of the tick, improving the larvicidal effect of the pyrethroid.

Although the larvae phase is widely used to evaluate the acaricidal activity of compounds derived from plants *in vitro*, the efficacy of the compounds can vary according to the developmental phase of the tick (Rosado-Aguilar et al., 2017). For instance, the wax layer is thicker in adults than in larvae, increasing the sequestration of compounds within the wax and reducing their efficacy (Adenubi et al., 2018). This study demonstrated the increase of anti-larval activity of cypermethrin by anonaine. Despite the protective effects against larva, the cypermethrin-anonaine combination needs improvement to increase activity against all life stages of the tick.

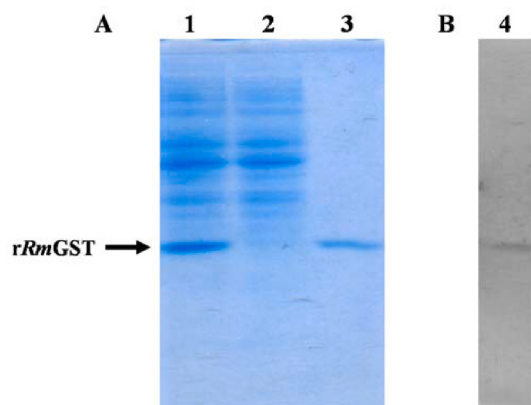


Fig. 3. A) SDS-PAGE (12% gel, with electrophoresis performed under reducing conditions) and B) Western blot of recombinant *R. microplus* GST. 1) Extract of *E. coli* cells expressing rRmGST; 2) Unbound fraction eluted in GSH chromatography; 3) Purified GST (rRmGST); 4) Western blot with anti-GST serum.

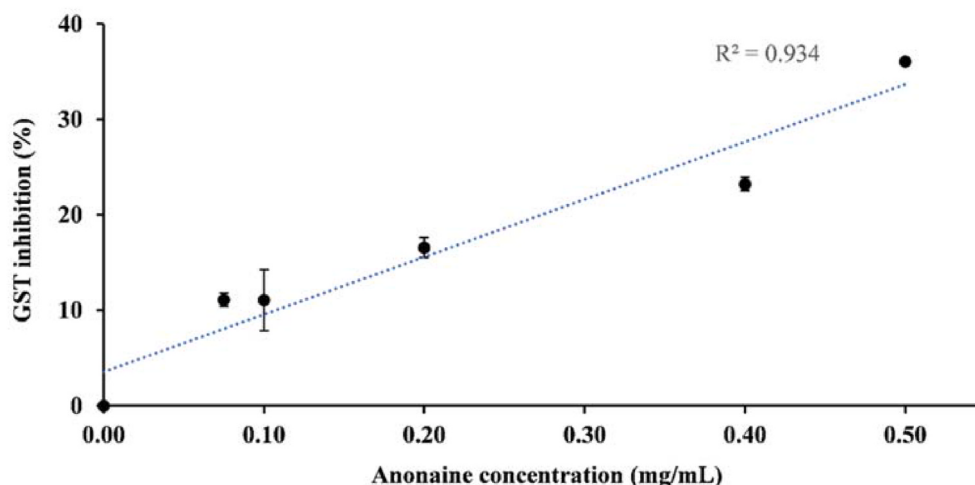


Figura 4. Inhibition curve for the anonaine on rRmGST. Y-axis: percentage of GST inhibition; X-axis: anonaine concentration in mg/mL.

Table 1

Effect of anonaine, cypermethrin, and their combination on larvae and engorged females of *Rhipicephalus microplus*.

Treatment	Larval immersion test			Adult immersion test		
	LC ₅₀ (mg/mL)	CI 95%	R ²	% Rovip	% Rhatch	C%
Anonaine (0.1 mg/mL) ^a	–	–	–	3.73 ± 9.90 ^a	–	6.24 ± 8.74 ^a
Anonaine (0.5 mg/mL) ^a	–	–	–	17.69 ± 24.10 ^a	37.4 ± 7.1 ^a	14.26 ± 25.82 ^a
Cypermethrin (CYP)	0.044 ^a	0.038–0.050	0.96	62.25 ± 6.76 ^b	96.85 ± 0.60 ^b	98.85 ± 0.30 ^b
CYP + anonaine (0.1 mg/mL)	0.057 ^b	0.054–0.061	0.99	52.42 ± 15.39 ^b	98.65 ± 6.97 ^b	99.44 ± 0.28 ^b
CYP + anonaine (0.5 mg/mL)	0.022 ^c	0.016–0.029	0.93	61.25 ± 2.68 ^b	94.27 ± 6.97 ^b	97.79 ± 2.60 ^b

^a Anonaine had no effect on larvae; LC₅₀: Lethal concentration (mg/mL) for 50% of individuals; CI: 95% confidence interval; R²: Regression Correlation Coefficient. % Rovip: Percentage of reduction in oviposition; % Rhatch: Percentage of hatching reduction; C%: Control percentage. Mean ± standard deviation. The same superscript letter in the same column indicates that the mean does not differ significantly at $p < 0.05$.

5. Conclusion

This study shows, *in silico* and *in vitro*, the capacity of anonaine to inhibit the rRmGST activity. The immersion tests revealed that anonaine can increase the toxic activity of cypermethrin against *R. microplus* larvae.

CRediT authorship contribution statement

Wallyson André dos Santos Bezerra: Investigation, Methodology, Formal analysis, Writing – original draft. **Caio Pavão Tavares:** Methodology, Formal analysis, Investigation. **Cláudia Quintino da Rocha:** Resources, Investigation, Writing – review & editing. **Itabajara da Silva Vaz Junior:** Resources, Investigation, Writing – review & editing. **Paul A.M. Michels:** Conceptualization, Supervision, Validation, Data curation, Formal analysis, Writing – review & editing. **Livio Martins Costa Junior:** Resources, Supervision, Writing – review & editing, Validation. **Alexandra Martins dos Santos Soares:** Conceptualization, Resources, Funding acquisition, Writing – review & editing, Validation, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exppara.2022.108398>.

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