Revista Eletrônica
Acervo Saúde
Electronic Journal Collection Health ISSN 2178-2091

Virtual screening of natural products as potential inhibitors of triosephosphate isomerase of *Rhipicephalus microplus*

Triagem virtual de produtos naturais como potenciais inibidores da triosefosfato isomerase de *Rhipicephalus microplus*

Evaluación virtual de productos naturales como posibles inhibidores de la triosafosfato isomerasa de *Rhipicephalus microplus*

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ABSTRACT

Objective: In this study, molecular docking of 332 natural products selected from the ZINC15 database was performed on the three-dimensional structure of *R. microplus* TIM using Molegro Virtual Docker. **Methods:** The absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of the best compounds were predicted. After molecular docking, the 20 compounds with the lowest MolDock Score, indicative of the highest predicted affinity to *R. microplus* TIM, were evaluated. **Results:** Fifty percent are categorized as alkaloids and aminoglycosides, 20% as dipeptides and terpenoids. Eighty percent comprise anticancer and antimicrobial compounds. Paclitaxel, dirithromycin, toposar, natamycin, and cabazitaxel exhibited the highest affinities for *R. microplus* TIM, with MolDock scores of -171.258, -168.586, -149.368, -148.880, and -148.810, respectively. **Conclusion:** Expanding research into TIM inhibition and modifying the studied compounds could thus lead to the discovery of new acaricides. This study's findings enhance our understanding of TIM inhibition in ticks, confirming its druggability as a target for natural compounds and aiding in the development of strategies for improved tick control.

Keywords: Ticks, Druggability, TIM inhibition, Molecular docking.

RESUMO

Objetivo: Neste estudo, o docking molecular de 332 produtos naturais selecionados do banco de dados ZINC15 foi realizado na estrutura tridimensional de *R. microplus* TIM usando o Molegro Virtual Docker. **Métodos:** As propriedades de absorção, distribuição, metabolismo, excreção e toxicidade (ADMET) dos melhores compostos foram previstas. Após o docking molecular, os 20 compostos com o menor MolDock Score, indicativo da maior afinidade prevista para *R. microplus* TIM, foram avaliados. **Resultados:** Cinquenta por cento são categorizados como alcaloides e aminoglicosídeos, 20% como dipeptídeos e terpenoides. Oitenta por cento compreendem compostos anticâncer e antimicrobianos. Paclitaxel, diritromicina, toposar, natamicina e cabazitaxel exibiram as maiores afinidades para *R. microplus* TIM, com pontuações MolDock de -171,258, -168,586, -149,368, -148,880 e -148,810, respectivamente. **Conclusão:** Expandir a pesquisa sobre a inibição de TIM e modificar os compostos estudados pode, portanto, levar à descoberta de novos acaricidas. As descobertas deste estudo aumentam nossa compreensão da inibição de TIM em carrapatos, confirmando sua drogabilidade como um alvo para compostos naturais e auxiliando no desenvolvimento de estratégias para melhor controle de carrapatos.

Palavras-chave: Carrapatos, Drogabilidade, Inibição de TIM, Docagem molecular.

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SUBMETIDO EM: 8/2023 | **ACEITO EM: 9/2024** | **PUBLICADO EM: 12/2024**

RESUMEN

Objetivo: En este estudio, se realizó el acoplamiento molecular de 332 productos naturales seleccionados de la base de datos ZINC15 sobre la estructura tridimensional de *R. microplus* TIM utilizando Molegro Virtual Docker. **Métodos:** Fueron evaluadas las propiedades de absorción, distribución, metabolismo, excreción y toxicidad (ADMET) de los mejores compuestos. Después del acoplamiento molecular, se evaluaron los 20 compuestos con el puntaje MolDock más bajo, indicativo de la afinidad más alta predecida por *R. microplus* TIM. **Resultados:** El cincuenta por ciento se clasifica como alcaloides y aminoglucósidos, el 20% como dipéptidos y terpenos. El ochenta por ciento comprende compuestos anticancerígenos y antimicrobianos. Paclitaxel, diritromicina, toposar, natamicina y cabazitaxel mostraron las mayores afinidades por la TIM de R. microplus, con puntuaciones de MolDock de -171,258, -168,586, -149,368, -148,880 y -148,810, respectivamente. **Conclusión:** La ampliación de la investigación sobre la inhibición de la TIM y la modificación de los compuestos estudiados podría conducir al descubrimiento de nuevos acaricidas. Los hallazgos de este estudio mejoran nuestra comprensión de la inhibición de la TIM en garrapatas, lo que confirma su capacidad de ser un objetivo para los compuestos naturales y ayuda al desarrollo de estrategias para un mejor control de las garrapatas.

Palabras clave: Garrapatas, Farmacología, Inhibición de TIM, Acoplamiento molecular.

INTRODUCTION

The tick *Rhipicephalus microplus* is the most significant ectoparasite in tropical and subtropical regions, causing annual economic losses estimated at US$ 3.2 billion (GRISI L, et al., 2014). Its bite induces dermal damage, leading to local inflammation that negatively impacts the livestock leather industry. Moreover, it adversely affects animal weight gain, thereby reducing meat production (JONSSON N, 2006; Nicaretta JE, et al., 2023). Additionally, *R. microplus* serves as the primary vector for various cattle-borne pathogens, including *Babesia* spp. and *Anaplasma spp.* (MIRABALLES C, et al., 2019; MARQUES R, et al., 2020).

Predominantly, synthetic acaricides are used to control these ectoparasites. However, continuous and indiscriminate use of acaricides has led to the emergence of chemically resistant tick populations (KLAFKE G, et al., 2017; AGWUNOBI DO, et al., 2021; WALDMAN J, et al., 2023). Therefore, the search for novel compounds with different mechanisms of action to exert effective acaricidal activity against *R. microplus* is crucial. Numerous chemical compounds have been explored for their potential in parasite control strategies, particularly those targeting specific parasite enzymes (BEZERRA WAS, et al., 2022; SAPORITI T, et al., 2022; MALAK N, et al., 2023).

Given that triosephosphate isomerase (TIM) is an enzyme involved in glycolysis and gluconeogenesis, processes that play a critical role in carbon and energy metabolism (KUMAR K, et al., 2012; LIANG N, et al., 2022), its potential as target for drug development against various parasites has been studied (BRAZ V, et al., 2019; JUÁREZ-SALDIVAR A, et al., 2021; GONZÁLEZ-MORALES LD, et al., 2023), including for TIM of *R. microplus* (SARAMAGO L, et al., 2018; MALAK N, et al., 2023). Despite the highly structural similarity of this enzyme between species, it has been possible to obtain selective inhibitors if they target the enzyme's dimer interface, as this region is poorly conserved (TÉLLEZ-VALENCIA A, et al., 2004).

Plant-derived products are attractive because of their generally low toxicity, limited environmental persistence, and complex chemical structure, which may hinder development of resistance resulting from modification of the compound (SELLES SMA, et al., 2021). Identifying and developing pharmaceuticals targeting essential tick enzymes, such as TIM, represent an innovative and promising approach to drug development.

In this context, natural products emerge as promising candidates for controlling *R. microplus* by targeting its TIM. Advancements in computational techniques have enabled the introduction of new virtual screening methods, providing a faster and cheaper alternative to in vitro screening of libraries of drug-like compounds (JUÁREZ-SALDIVAR A, et al., 2021).

These methods rely on the in-silico analysis and modeling of molecular interactions between potential ligands and target molecules (WADOOD A, et al., 2013; KIAMETIS et al., 2017), significantly contributing to

the identification of novel drug candidates (CHOUBEY SK, JEYARAMAN J, 2016; SARAMAGO L, et al., 2018; GANESAN M, et al., 2020). Given the scientific and economic significance of advancing novel acaricidal products against ticks, coupled with the pivotal role of TIM as a target enzyme essential to tick physiology, this study utilized in-silico techniques to evaluate potential natural products against *R. microplus* TIM.

METHODS

Structure of natural products and ADMET features

The structures of 332 compounds were obtained, in mol2 format, from the ZINC15 database using the filters: natural products, and for sale and world (approved drugs in major jurisdictions, including the FDA, i.e. DrugBank approved). The theoretical ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of compounds were analyzed using PreADMET software (KWANG LS, 2005).

Molecular docking of natural products onto triosephosphate isomerase (TIM) of *Rhipicephalus microplus*

To analyze the potential inhibitory activity of selected compounds to the *R. microplus* enzyme, molecular docking was carried out in Loop 3 of the dimer interface of the tick TIM, using Molegro Virtual Docker 6.0 (MVD) software. The structure of the TIM, at 2.40 Å resolution (PDB ID: 3TH6 – chain A), was obtained from the Protein Data Bank (www.rcsb.org).

The structures of natural products and TIM were imported into the MVD workspace in 'mol2' format. The enzyme structure was 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 center coordinates X: 9.52; Y: 5.94; Z: -23.62 Å, using MolDock Score [GRID] as the score function and the MolDock SE as the search algorithm. Ten independent runs were conducted, and the results were expressed in the MolDock score. The more negative the number, the better the binding.

The 20 compounds exhibiting the lowest MolDock Scores were identified, and their scaffold, chemical classes, and biological classes were determined through analysis using the ZINC15 database. Subsequently, the five molecules with the lowest MolDock scores were selected for further examination. The optimal pose of each molecule with TIM was visualized and assessed utilizing the PyMOL Molecular Graphics System v1.3 (http://www.pymol.org). Additionally, the residues of TIM interacting with natural products were analyzed using the Discovery Studio Visualizer program v21.1.0 (https://www.3ds.com/products/biovia/discovery-studio).

RESULTS

Molecular docking

After molecular docking of structures of 322 natural products, selected from the ZINC15 database, onto the available, crystallographically-determined three-dimensional structure of *R. microplus* TIM, as described in the Methodology section, the 20 compounds with the lowest MolDock scores were retained (listed in Table 1). Alkaloids, aminoglycosides, dipeptides, and terpenoids are the predominant classes comprising these compounds, representing 25%, 25%, 10%, and 10%, respectively. Furthermore, regarding their biological activities, 80% is represented by compounds with anticancer and antimicrobial activities. All compounds contain aromatic rings in their scaffolds, as shown in Supplementary (**Figure 1**).

Additionally, all compounds have -O-, -N-, and/or -NH- groups in their structures. Considering the molecules with the best predicted affinities to *R. microplus* TIM, the MolDock scores for paclitaxel, dirithromycin, toposar, natamycin, and cabazitaxel were -171.258, -168.586, -149.368, -148.880, and -148.810, respectively (**Table 1**). These compounds commonly interact with the residues Tyr67, Val69, Glu70, Gln71, Phe74, and Met82 of TIM (**Figure 1**). The physicochemical characteristics and predicted ADMET properties of these natural products are shown in (**Table 2)**.

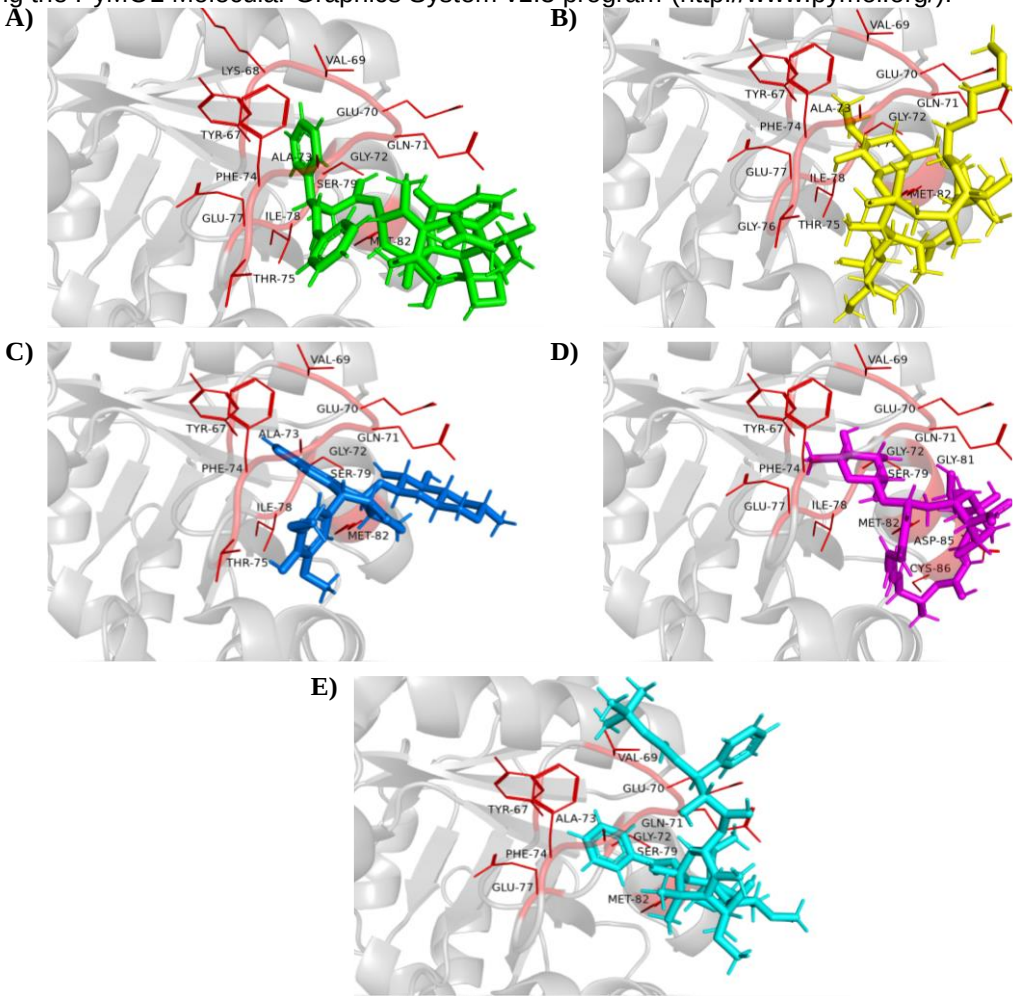
Paclitaxel, dirithromycin, toposar, natamycin, and cabazitaxel present molecular weights of 853.330, 834.550, 588.180 665.300, and 835.380 g/mol, respectively. All compounds have octanol/water partition coefficient (LogP) values below 5.0. Only paclitaxel, toposar, and cabazitaxel are predicted to have high human intestinal absorption (83.692%, 92.199%, and 91.281%, respectively). Natamycin showed positive Ames test mutagenicity. Furthermore, it was predicted that the compounds could enter the brain, as the values for their distribution across the blood-brain barrier (BBB) were 0.024, 0.044, 0.037, 0.045, and 0.023, for paclitaxel, dirithromycin, toposar, natamycin, and cabazitaxel, respectively.

Table 1- Virtual screening results showing the highest ranked compounds based on MolDock score in triosephosphate isomerase of *Rhipicephalus microplus*.

Name	Zinc code	Biological activity	Chemical class	Molecule formula	MolDock score
Paclitaxel	96006020	Anticancer	Diterpenoid	$C_{47}H_{51}NO_{14}$	-171.258
Dirithromycin	96095661	Antibiotic	Aminoglycoside	$C_{42}H_{78}N_2O_{14}$	-168.586
Toposar	3938684	Anticancer	Podophyllotoxin	$C_{29}H_{32}O_{13}$	-149.368
Natamycin	253633751	Antifungal	Aminoglycoside	$C_{33}H_{47}NO_{13}$	-148.880
Cabazitaxel	85536932	Anticancer	Diterpenoid	$C_{45}H_{57}NO_{14}$	-148.810
Dronedarone	49933061	Antiarrhythmic	Aryl-phenylketones	$C_{31}H_{44}N_2O_5S$	-144.797
Cytisine	1599729	Help with smoking cessation	Alkaloid	$C_{11}H_{14}N_2O$	-142.495
Hyperforin	4097413	Antidepressant/Anxiolytic	Bicyclic monoterpenoid	$C_{35}H_{52}O_4$	-141.420
Vinblastine	85555528	Anticancer	Alkaloid	$C_{46}H_{58}N_4O_9$	-140.789
Vindesine	8214470			$C_{43}H_{55}N_5O_7$	-138.474
Synribo	43450324			$C_{29}H_{39}NO_9$	-135.033
Oxacillin	3875439	Antibiotic	Dipeptide	$C_{19}H_{19}N_3O_5S$	-134.530
Docetaxel	85537053	Anticancer	Diterpenoid	$C_{43}H_{53}NO_{14}$	-134.288
Aztreonam	3830264	Antibiotic	Monobactam	$C_{13}H_{17}N_5O_8S_2$	-133.567
Cloxacillin	3875417		Dipeptide	$C_{19}H_{18}ClN_3O_5S$	-131.394
Vincristine	85432549	Anticancer	Alkaloid	$C_{46}H_{56}N_4O_{10}$	-130.217
Paromomycin	60183170	Antibiotic	Aminoglycoside	$C_{23}H_{45}N_5O_{14}$	-129.937
Erythromycin	85534336			$C_{37}H_{67}NO_{13}$	-129.235
Steviolbioside	79216653	Antiviral	Glycoside	$C_{32}H_{50}O_{13}$	-129.203
Streptomycin	8214681	Antibiotic	Aminoglycoside	$C_{21}H_{39}N_7O_{12}$	-129.188

Source: Bezerra WAS, et al., 2024.

Figure 1- Cartoon representation of triosephosphate isomerase (TIM) of *Rhipicephalus microplus* (PDB ID: 3TH6) in complex with A) paclitaxel; B) dirithromycin; C) toposar; D) natamycin; and E) cabazitaxel. The ligands are shown as sticks. In red, TIM residues within 3.5 Å of the natural product. The best pose of TIM with each ligand was visualized and analyzed using the PyMOL Molecular Graphics System v1.3 program (http://www.pymol.org/).



Source: Bezerra WAS, et al., 2024.

Table 2- Physicochemical and ADMET properties predicted for natural compounds.

ID	Paclitaxel	Dirithromycin	Toposar	Natamycin	Cabazitaxel
Molecular weight (g/mol)	853.330	834.550	588.180	665.300	835.380
Hydrogen-bond acceptors	15	16	13	14	15
Hydrogen-bond donors	4	5	3	8	3
LogP	3.580	3.684	1.387	0.091	4.432
Caco2	20.426	41.428	19.140	14.279	23.023
HIA	91.281	66.365	83.692	20.963	92.199
MDCK	0.0434	0.0434	0.0567	0.0437	0.0434
PGP_inh	Non	Inhibitor	Non	Inhibitor	Non
PPB	86.187	14.557	56.392	33.038	82.401
PWS (mg/L)	0.0031	79.197	12.196	52.085	0.0168
Skin_Permeability	-1.834	-3.601	-4.655	-2.770	-1.467
BBB	0.024	0.044	0.037	0.045	0.023
CYP_2C19_inh	Non	Non	Inhibitor	Inhibitor	Non
CYP_2C9_inh	Inhibitor	Non	Inhibitor	Inhibitor	Non
CYP_2D6_inh	Non	Inhibitor	Non	Inhibitor	Non
CYP_2D6_sub	Non	Weakly	Non	Non	Non
CYP_3A4_inh	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP_3A4_sub	Substrate	Substrate	Substrate	Weakly	Substrate
Algae_at	0.00039	0.00031	0.01727	0.00354	0.00054
Ames_test	Non-mutagen	Non-mutagen	Non-mutagen	Mutagen	Non-mutagen
Carcino_Mo	Positive	Negative	Negative	Positive	Negative
Carcino_Rat	Negative	Negative	Negative	Positive	Negative
daphnia_at	0.01371	0.10608	0.29523	0.92061	0.01212
hERG_inh	Ambiguous	Ambiguous	Ambiguous	Ambiguous	Low_risk
Medaka_at	0.0007	0.0277	0.1803	1.769	0.0005
Minnow_at	0.0051	0.0303	0.2889	3.478	0.0029

Note: BBB – Blood-Brain Barrier (C.brain/C.blood); Caco-2 – Caco2-cell model; HIA – Human Intestinal Absorption model (HIA, %); MDCK – Madin-Darby Canine Kidney (nm/sec); PGP_inhibition – P-glycoprotein inhibitor; PPB – Plasma Protein Binding (%); PWS – Pure water solubility (mg/L); Skin Permeability- Skin permeability in cm/hour. Algae at – algae test (mg/L); Ames Test – Ames Salmonella; CYP – Cytochrome P450; Carcino M – carcinogenesis test in the mouse; Carcino R – carcinogenesis test in rats; Daphnia at – test on crustacean Daphnia; hERG inhib. – hERG-controlled potassium channel inhibition; Medaka_at – test on medaka fish; Minnow_at – test on small freshwater fish.
Source: Bezerra WAS, et al., 2024.

DISCUSSION

Synthetic acaricides are extensively utilized in veterinary and human medicine for parasitic disease control. However, the emergence of resistance underscores the need for alternative approaches (OBAID MK, et al., 2022). Exploration of new bioactive compounds with enhanced potency and selectivity for tick targets offers potential solutions to these challenges. In silico methods such as molecular docking facilitate the discovery of novel compounds that bind to parasites' molecular targets (RUYCK J, et al., 2016).

Triosephosphate isomerase (TIM) is an enzyme involved in both glycolysis and gluconeogenesis where it catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. To produce selective inhibitors, it appeared to be essential to target the enzyme's dimer interface, which exhibits poor conservation between species, in contrast to the enzyme's overall structure which is well conserved (TÉLLEZ-VALENCIA A, et al., 2004).

In this study, molecular docking of natural products from the ZINC15 database onto the *R. microplus* TIM protein was conducted. Docking analysis predicts the optimal molecular orientation for binding of a compound to a protein and calculates the corresponding binding energy (MALAK N, et al., 2023). The 20 compounds with the best MolDock scores (**Table 1**), including paclitaxel, dirithromycin, toposar, natamycin, and cabazitaxel, were suggested as potential TIM ligands targeting the protein's dimer interface what is expected to lead to inhibition since a dimeric structure is required for activity (NÁJERA H, et al., 2003).

Although these compounds have antiprotozoal or antimicrobial activity (BENAIM G, et al., 2014; PENSEL PE, et al., 2014; AWASTHI BP, MITRA K, 2018; PICHKUR EB, et al., 2020; CAO Y, et al., 2023), to the best of our knowledge, none has yet been described in the literature as having activity against ticks. However,

alkaloids and terpenes, two of the classes to which compounds belong that were identified in the present study, have been reported to possess anti-tick activity (CARROLL JF, et al., 2007; LIMA HG, et al., 2020; CARDOSO AS, et al., 2020; SILVA GD, et al., 2021).

In medicinal chemistry, similar molecules exhibit similar biological effects, guiding the modification of active compounds. Bioisosteric replacements transform lead structures into enzyme inhibitors, receptor agonists/antagonists, and other active agents. This systematic replacement strategy enables to optimize drug properties, yielding a variety of therapeutically effective medications (KUBINYI H, 2002; POUPAERT J, et al., 2005).

However, it needs to be noted that several surprising structure-activity relationships demonstrate that chemically similar compounds may exhibit significantly different biological actions and activities (DINARVAND M, SPAIN M, 2021; ALIZADEH SR, EBRAHIMZADEH MA, 2022; EBETINO FH, et al., 2022; SHAMSUDIN NF, et al., 2022). It is important to highlight that the structures identified in the present study have functional groups in common that could be explored in the early stages of drug design to develop novel TIM inhibitors. As shown in **Supplementary Figure 1**, all compounds have -O-, and/or -N- / -NH- groups.

The selected compounds' significant delocalized conjugated structures play a pivotal role in exerting pharmacological activity (KAUR R, KUMAR K, 2021; LIU R, et al., 2022). Moreover, interactions between aromatic and heteroaromatic rings are major contributors to protein structure and protein–ligand complexation (FASAN R, et al., 2006; SALONEN LM, et al., 2011). In this study, the 20 selected compounds contain aromatic rings. Previous reports have shown TIM inhibitors that bind to the interface through aromatic interactions (JUÁREZ-SALDIVAR A, et al., 2021; KURKCUOGLU Z, et al., 2015).

In this study, TIM residues within a proximity of 3.5 Å to the compounds were evaluated, a distance suggested as ideal for interaction (LIU Z, et al., 2008; BIANCHI V, et al., 2012). The residues Tyr67, Val69, Glu70, Gln71, Gly72, Phe74, Ser79, and Met82 in *R. microplus* TIM may be key residues involved in the interaction, as they are all within 3.5 Å of each of the five selected compounds (Figure 1). These compounds—paclitaxel, dirithromycin, toposar, natamycin, and cabazitaxel—bind to the TIM dimer interface at residues in Loop 3 (**Figure 1**).

Considering that Loop 3 residues are involved in hydrogen-bond interactions with Loop 1 of the other subunit, contributing to the integrity of the dimer (SARAMAGO L, et al., 2018), it is suggested that these compounds could act by perturbing the interface region, leading to dimer rupture, similar as reported for other TIM inhibitors (OLIVARES-ILLANA V, et al., 2007; OLIVER C, TIMSON DJ, 2017; SARAMAGO L, et al., 2018; VÁZQUEZ-JIMÉNEZ LK, et al., 2022).

Saramago et al. (2018) demonstrated that a compound from the benzofuroxan family, with an IC_{50} on *R. microplus* TIM of 49 µM, binds to the dimer interface, interacting with residues on Loop 3 (Glu70, Gln71, Ser79, and Met82), as observed for the interaction of the aforementioned compounds (**Figure 1**). Although TIM is also an essential enzyme in mammals, only natural products approved for human use were selected to minimize the risk of mammalian toxicity. The physicochemical parameters and predicted ADMET properties of natural products were analyzed using the PreADMET tool (**Table 2**).

None of the compounds comply with Lipinski's rule of five, a guiding principle for assessing drug likeness and designing chemical compounds for potential oral activity (LIPINSKI CA, et al., 1997; RAJALAKSHMI R, et al., 2021). However, it is worth noting that many natural products which don't comply with Lipinski's rule criteria are still capable of traversing cell membranes (LEESON PD, DAVIS AM, 2004; O'SHEA R, MOSER HE, 2008; ABDELMOHSEN UR, et al., 2017).

For instance, the functional mechanisms of paclitaxel primarily involve inhibiting the dynamics of the microtubule spindle, thereby controlling cell proliferation and DNA repair (KHANNA C, et al., 2015; YAN-HUA Y, et al., 2020). Based on the findings of this study, the investigated natural products may serve as potential inhibitors of the TIM enzyme in *R. microplus* or as lead compounds for the design of new inhibitors. Expanding research into TIM inhibition and further modifying or synthesizing compounds based on these scaffolds could enhance their efficacy, potentially leading to their application as acaricides. This could significantly contribute to the development of innovative anti-tick drugs.

CONCLUSION

In this study, virtual screening based on molecular docking was employed to identify natural compounds among FDA-approved drugs that are predicted to bind to TIM from *R. microplus* and interfere with its activity. These findings offer promise for the development of novel strategies to combat acaricide-resistant cattle ectoparasites, potentially mitigating economic losses in the livestock industry.

ACKNOWLEDGMENT

This study was supported in part by Maranhão State Research Foundation (FAPEMA) - INFRA 03170/18, UNIVERSAL 00869/22 and FAPEMA IECT Biotechnology/Financier of Studies and Projects (FINEP) process 2677/17. It was also financed by the Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001). We also thank CAPES for awarding a fellowship to W.A.S Bezerra.

Declaration of artificial intelligence (AI) and AI-assisted technologies in the writing process: During the preparation of this work, the authors used ChatGPT to enhance the cohesion of an initial version of the manuscript. After using this tool/service, the authors reviewed and edited the text as needed and take full responsibility for the content of the publication.

REFERENCES

1. ABDELMOHSEN UR, et al. Potential of marine natural products against drug-resistant fungal, viral, and parasitic infections. The Lancet Infectious Diseases. 2017; 17(2): 30-41.
2. AGWUNOBI DO, et al. A retrospective review on ixodid tick resistance against synthetic acaricides: implications and perspectives for future resistance prevention and mitigation. Pesticide Biochemistry Physiology. 2021; 173: 1-17.
3. ALIZADEH SR, EBRAHIMZADEH MA. O-Glycoside quercetin derivatives: Biological activities, mechanisms of action, and structure–activity relationship for drug design, a review. Phytotherapy Research. 2022; 36(2): 778-807.
4. AWASTHI BP, MITRA K. In vitro leishmanicidal effects of the anti-fungal drug natamycin are mediated through disruption of calcium homeostasis and mitochondrial dysfunction. Apoptosis. 2018;23(7): 420-35.
5. BENAIM G, et al. Dronedarone, an amiodarone analog with improved anti-Leishmania mexicana efficacy. Antimicrobial agents chemotherapy. 2014; 58(4): 2295-303.
6. BEZERRA WAS, et al. Anonaine from Annona crassiflora inhibits glutathione S-transferase and improves cypermethrin activity on Rhipicephalus (Boophilus) microplus (Canestrini, 1887). Experimental Parasitology. 2022; 243: 1-8.
7. BIANCHI V, et al. Identification of binding pockets in protein structures using a knowledge-based potential derived from local structural similarities. BioMed Central Bioinformatics. 2012; 13: 1-13.
8. BRAZ V, et al. Inhibition of energy metabolism by 3-bromopyruvate in the hard tick Rhipicephalus microplus. Comparative Biochemistry Physiology Part C: Toxicology Pharmacology. 2019; 218: 55-61.
9. CAO Y, et al. Study on the Antifungal Activity and Potential Mechanism of Natamycin against Colletotrichum fructicola. Journal of Agricultural Food Chemistry. 2023; 71(46): 17713-22.
10. CARDOSO AS, et al. Terpenes on Rhipicephalus (Boophilus) microplus: Acaricidal activity and acetylcholinesterase inhibition. Veterinary parasitology. 2020; 280: 1-5.
11. CARROLL JF, et al. Repellency of two terpenoid compounds isolated from Callicarpa americana (Lamiaceae) against Ixodes scapularis and Amblyomma americanum ticks. Experimental applied acarology. 2007; 41: 215-24.
12. CHOUBEY SK, JEYARAMAN J. A mechanistic approach to explore novel HDAC1 inhibitor using pharmacophore modeling, 3D-QSAR analysis, molecular docking, density functional and molecular dynamics simulation study. Journal of Molecular Graphics Modelling. 2016; 70: 54-69.
13. DINARVAND M, SPAIN M. Identification of bioactive compounds from marine natural products and exploration of Structure-Activity Relationships (SAR). Antibiotics. 2021; 10(3): 1-24.
14. EBETINO FH, et al. Bisphosphonates: The role of chemistry in understanding their biological actions and structure-activity relationships, and new directions for their therapeutic use. Bone. 2022; 156: 116289.
15. FASAN R, et al. Structure–activity studies in a family of β-Hairpin protein epitope mimetic inhibitors of the p53–HDM2 protein–protein interaction. Chemistry biology Chemistry. 2006; 7(3): 515-26.
16. GANESAN M, et al. Design, synthesis, α-amylase inhibition and in silico docking study of novel quinoline bearing proline derivatives. Journal of Molecular Structure. 2020; 1208: 1-51.

17. GONZÁLEZ-MORALES LD, et al. Triose phosphate isomerase structure-based virtual screening and in vitro biological activity of natural products as Leishmania mexicana inhibitors. Pharmaceutics. 2023; 15(8): 1-17.
18. GRISI L, et al. Reassessment of the potential economic impact of cattle parasites in Brazil. Revista Brasileira de Parasitologia Veterinária. 2014; 23: 150-6.
19. JONSSON N. The productivity effects of cattle tick (Boophilus microplus) infestation on cattle, with particular reference to Bos indicus cattle and their crosses. Veterinary parasitology. 2006; 137(1-2): 1-10.
20. JUÁREZ-SALDIVAR A, et al. Virtual screening of fda-approved drugs against triose phosphate isomerase from Entamoeba histolytica and Giardia lamblia identifies inhibitors of their trophozoite growth phase. International Journal of Molecular Sciences. 2021; 22(11): 1-8.
21. KAUR R, KUMAR K. Synthetic and medicinal perspective of quinolines as antiviral agents. European Journal of Medicinal Chemistry. 2021; 215: 1-39.
22. KHANNA C, et al. A review of paclitaxel and novel formulations including those suitable for use in dogs. Journal of veterinary internal medicine. 2015; 29(4): 1006-12.
23. KIAMETIS AS, et al. Potential acetylcholinesterase inhibitors: molecular docking, molecular dynamics, and in silico prediction. Journal of molecular modeling. 2017; 23: 1-1.
24. KLAFKE G, et al. Multiple resistance to acaricides in field populations of Rhipicephalus microplus from Rio Grande do Sul state, Southern Brazil. Ticks tick-borne diseases. 2017; 8(1): 73-80.
25. KUBINYI H. Chemical similarity and biological activities. Journal of the Brazilian Chemical Society. 2002; 13(6): 717-26.
26. KUMAR K, et al. Cloning, overexpression and characterization of Leishmania donovani triosephosphate isomerase. Experimental parasitology. 2012; 130(4): 430-6.
27. KURKCUOGLU Z, et al. How an inhibitor bound to subunit interface alters triosephosphate isomerase dynamics. Biophysical Journal. 2015; 109(6): 1169-78.
28. KWANG LS. Editor In silico high-throughput screening for ADME/Tox properties: PreADMET program. Abstr Conf Comb Chem Jpn, 2005.
29. LEESON PD, DAVIS AM. Time-related differences in the physical property profiles of oral drugs. Journal of medicinal chemistry. 2004; 47(25): 6338-48.
30. LIANG N, et al. Characterization and evaluation of a new triosephosphate isomerase homologue from Haemaphysalis longicornis as a candidate vaccine against tick infection. Ticks Tick-borne Diseases. 2022; 13(4): 1-7.
31. LIMA HG, et al. Anti-tick effect and cholinesterase inhibition caused by Prosopis juliflora alkaloids: in vitro and in silico studies. Revista Brasileira de Parasitologia Veterinária. 2020; 29: 1-15.
32. LIPINSKI CA, et al. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced drug delivery reviews. 1997; 23(1-3): 3-25.
33. LIU R, et al. Research progress on the biological activities of metal complexes bearing polycyclic aromatic hydrazones. Molecules. 2022; 27(23): 1-56.
34. LIU Z, et al. Geometrical Preferences of the Hydrogen Bonds on Protein− Ligand Binding Interface Derived from Statistical Surveys and Quantum Mechanics Calculations. Journal of Chemical Theory Computation. 2008; 4(11): 1959-73.
35. MALAK N, et al. Density Functional Theory Calculations and Molecular Docking Analyses of Flavonoids for Their Possible Application against the Acetylcholinesterase and Triose-Phosphate Isomerase Proteins of Rhipicephalus microplus. Molecules. 2023; 28(8): 1-20.
36. MARQUES R, et al. Climate change implications for the distribution of the babesiosis and anaplasmosis tick vector, Rhipicephalus (Boophilus) microplus. Veterinary research. 2020; 51: 1-10.
37. MIRABALLES C, et al. Rhipicephalus microplus, babesiosis and anaplasmosis in Uruguay: current situation and control or elimination programs on farms. Experimental Applied Acarology. 2019; 78: 579-93.
38. NÁJERA H, et al. Thermodynamic characterization of yeast triosephosphate isomerase refolding: insights into the interplay between function and stability as reasons for the oligomeric nature of the enzyme. Biochemical Journal. 2003; 370(3): 785-92.
39. NICARETTA JE, et al. Selective versus strategic control against Rhipicephalus microplus in cattle: A comparative analysis of efficacy, animal health, productivity, cost, and resistance management. Veterinary Parasitology. 2023; 321: 1-16.
40. O'SHEA R, MOSER HE. Physicochemical properties of antibacterial compounds: implications for drug discovery. Journal of medicinal chemistry. 2008; 51(10): 2871-8.
41. OBAID MK, et al. Acaricides resistance in ticks: selection, diagnosis, mechanisms, and mitigation. Frontiers in Cellular Infection Microbiology. 2022; 12: 1-20.

42. OLIVARES-ILLANA V, et al. Perturbation of the dimer interface of triosephosphate isomerase and its effect on Trypanosoma cruzi. PLoS neglected tropical diseases. 2007; 1(1): 1-8.
43. OLIVER C, TIMSON DJ. In silico prediction of the effects of mutations in the human triose phosphate isomerase gene: towards a predictive framework for TPI deficiency. European Journal of Medical Genetics. 2017; 60(6): 289-98.
44. PENSEL PE, et al. In vitro effect of 5-fluorouracil and paclitaxel on Echinococcus granulosus larvae and cells. Acta tropica. 2014; 140: 1-9.
45. PICHKUR EB, et al. Insights into the improved macrolide inhibitory activity from the high-resolution cryo-EM structure of dirithromycin bound to the E. coli 70S ribosome. RNA. 2020; 26(6): 715-23.
46. POUPAERT J, et al. 2 (3H)-benzoxazolone and bioisosters as “privileged scaffold” in the design of pharmacological probes. Current medicinal chemistry. 2005; 12(7): 877-85.
47. RAJALAKSHMI R, et al. In silico studies: Physicochemical properties, drug score, toxicity predictions and molecular docking of organosulphur compounds against Diabetes mellitus. Journal of Molecular Recognition. 2021; 34(11): 1-21.
48. RUYCK J, et al. Molecular docking as a popular tool in drug design, an in silico travel. Advances Applications in Bioinformatics Chemistry. 2016: 1-11.
49. SALONEN LM, et al. Aromatic rings in chemical and biological recognition: energetics and structures. Angewandte Chemie International Edition. 2011; 50(21): 4808-42.
50. SAPORITI T, et al. Phenotypic and Target-Directed Screening Yields New Acaricidal Alternatives for the Control of Ticks. Molecules. 2022; 27(24): 1-14.
51. SARAMAGO L, et al. Novel and selective Rhipicephalus microplus triosephosphate isomerase inhibitors with acaricidal activity. Veterinary Sciences. 2018; 5(3): 1-19.
52. SELLES SMA, et al. Acaricidal and repellent effects of essential oils against ticks: a review. Pathogens. 2021; 10(11): 1-17.
53. SHAMSUDIN NF, et al. Antibacterial effects of flavonoids and their structure-activity relationship study: A comparative interpretation. Molecules. 2022; 27(4): 1-43.
54. SILVA GD, et al. In vitro and in silico studies of the larvicidal and anticholinesterase activities of berberine and piperine alkaloids on Rhipicephalus microplus. Ticks Tick-borne Diseases. 2021; 12(2): 1-6.
55. TÉLLEZ-VALENCIA A, et al. Inactivation of triosephosphate isomerase from Trypanosoma cruzi by an agent that perturbs its dimer interface. Journal of molecular biology. 2004; 341(5): 1355-65.
56. VÁZQUEZ-JIMÉNEZ LK, et al. Ligand-based virtual screening and molecular docking of benzimidazoles as potential inhibitors of triosephosphate isomerase identified new trypanocidal agents. International Journal of Molecular Sciences. 2022; 23(17): 1-27.
57. WADOOD A, et al. In-silico drug design: An approach which revolutionarised the drug discovery process. Drug design and delivery. 2013;1(1):1-4.
58. WALDMAN J, et al. Putative target sites in synganglion for novel ixodid tick control strategies. Ticks Tick-borne Diseases. 2023; 14(3): 1-12.
59. YAN-HUA Y, et al. Research progress on the source, production, and anti-cancer mechanisms of paclitaxel. Chinese journal of natural medicines. 2020; 18(12): 890-7.

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Medicinal Chemistry, **2024**, *20*, 912-919

RESEARCH ARTICLE

In silico and *In vitro* Assessment of Dimeric Flavonoids (Brachydins) on *Rhipicephalus microplus* Glutathione S-transferase

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ARTICLE HISTORY

Received: December 14, 2023
Revised: February 26, 2024
Accepted: March 13, 2024

DOI:
10.2174/0115734064298481240517072216

Abstract: ***Introduction:*** *Rhipicephalus microplus*, an important cattle ectoparasite, is responsible for a substantial negative impact on the economy due to productivity loss. The emergence of resistance to widely used commercial acaricides has sparked efforts to explore alternative products for tick control.

Methods: To address this challenge, innovative solutions targeting essential tick enzymes, like glutathione S-transferase (GST), have gained attention. Dimeric flavonoids, particularly brachydins (BRAs), have demonstrated various biological activities, including antiparasitic effects. The objectives of this study were to isolate four dimeric flavonoids from *Fridericia platyphylla* roots and to evaluate their potential as inhibitors of *R. microplus* GST.

Results: *In vitro* assays confirmed the inhibition of *R. microplus* GST by BRA-G, BRA-I, BRA-J, and BRA-K with IC_{50} values of 0.075, 0.079, 0.075, and 0.058 mg/mL, respectively, with minimal hemolytic effects. Molecular docking of BRA-G, BRA-I, BRA-J, and BRA-K in a three-dimensional model of *R. microplus* GST revealed predicted interactions with MolDock Scores of -142.537, -126.831, -108.571, and -123.041, respectively. Both *in silico* and *in vitro* analyses show that brachydins are potential inhibitors of *R. microplus* GST.

Conclusion: The findings of this study deepen our understanding of GST inhibition in ticks, affirming its viability as a drug target. This knowledge contributes to the advancement of treatment modalities and strategies for improved tick control.

Keywords: Tick, dimeric flavonoids, GST inhibition, *Fridericia platyphylla, Rhipicephalus microplus.*

1. INTRODUCTION

The cattle tick *Rhipicephalus microplus* (Canestrini 1887) (Acari: Ixodidae) is an important economic threat to livestock production due to its role as a vector for various pathogens, resulting in diseases of the cattle [1]. The effectiveness of synthetic acaricides in controlling *R. microplus* populations has been compromised by the development and subsequent spread of resistance [2, 3]. Numerous chemical compounds have undergone investigation for their potential use in strategies for parasite control, specifically to identify molecules that selectively target parasite enzymes to combat infections [4-7]. Glutathione S-transferase (GST) has emerged as a promising target for antiparasitic drug development, given its pivotal role in detoxifying harmful substances [8]. Inhibitors capable of disrupting the GST activity in ticks, thereby interfering with their detoxification system, represent a promising and innovative alternative for parasite control [5].

Plants have evolved defense mechanisms against pests through the production of a wide range of phytochemicals, which are currently under investigation as potential alterna-

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1875-6638/24 $65.00+.00

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Medicinal Chemistry

tives for tick control [9, 10]. Plant products are particularly studied due to their low toxicity, scarce environmental permanence, and the complex chemistry that hinders the development of the resistances. They are considered potential alternatives even for managing ticks that display resistance to traditional acaricides [11].

Notably, flavonoids have demonstrated activity against ticks [12, 13]. Furthermore, flavonoids are well-recognized among natural compounds for their capacity to inhibit GST [5, 14, 15]. Within this context, the unusual dimeric flavonoids known as brachydins, isolated from the roots of the plant species *Fridericia platyphylla* (Cham.) L.G. Lohmann (syn: *Arrabidaea brachypoda* Bureau*,* Bignoniaceae*)*, have emerged as bioactive compounds with several biological properties, including activity against the human endoparasitic protists *Trypanosoma cruzi* and *Leishmania amazonensis* [16, 17]. Considering this context, brachydins represent promising candidates for the control of *R. microplus* by targeting its GST.

Recently, computational techniques have facilitated the discovery of new drug candidates [18-21]. For instance, employing molecular docking, in which the favored binding pose of a candidate ligand on a structural model of a macromolecular target is predicted, allows the identification of potential drug candidates.

Given the scientific and economic significance of developing new compounds effective against *R. microplus*, and considering the lack of studies on the biological and/or antiparasitic activities of the brachydins BRA-G, BRA-I, BRA-J, and BRA-K, this study utilized *in silico* and *in vitro* evaluations to investigate the potential of these brachydins as inhibitors of tick GSTs.

2. MATERIALS AND METHODS

2.1. Extraction and Isolation of Brachydins G, I, J, and K

Brachydins (BRA-G, BRA-I, BRA-J, and BRA-K) were isolated, and their structures were defined as previously described [22]. Briefly, a crude ethanol extract prepared from *F. platyphylla* roots was evaporated to dryness. Further, after liquid/liquid extractions, a dichloromethane (CH_2Cl_2) and a hydromethanolic fraction were obtained and dried. The hydromethanolic fraction underwent further separation using medium-pressure liquid chromatography (MPLC). The resulting fractions were analyzed using an accurate, high-performance liquid chromatography–photodiode array (HPLC-PDA). BRA-G, BRA-I, BRA-J and BRA-K, corresponded to the peaks in fractions 7, 9, 10 and 11, respectively.

2.2. Recombinant GST of *Rhipicephalus microplus* (r*Rm*GST) and Inhibition of r*Rm*GST by Brachydins

The *R. microplus* recombinant GST (r*Rm*GST) was prepared as previously described [4, 23, 24]. The enzymatic activity of this GST was measured at 25°C in a VersaMax™ Microplate Reader and 96-well microplates, using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich, Saint Louis, MO, USA) and 3,4-dichloronitrobenzene (DCNB) (Sigma-Aldrich), as previously described [25]. Briefly, for the inhibition tests, brachydins (BRA-G, BRA-I, BRA-J, BRA-K) were diluted in 1% DMSO at 10 mg/mL (stock solution). GST inhibition by brachydins was assessed within a concentration range of 0.010–0.2 mg/mL. These inhibition tests were conducted using 10 µL (equivalent to 0.7 µg of protein) of recombinant protein. GST, CDNB, GSH, and DMSO (0.1%) were used as negative controls. Readings were performed at 340 nm for 15 min at 15 s intervals. The assays were performed in triplicate.

2.3. Hemolytic Activity Assessment of Brachydins on Bovine Erythrocytes

The *in vitro* hemolysis assay was carried out by measuring the lysis of bovine erythrocytes as previously described [26], with minor modifications. Bovine whole blood was collected and processed to obtain a suspension of red blood cells (RBC) in 0.15 M NaCl, which was then incubated with varying concentrations of brachydins (BRA-G, BRA-I, BRA-J, BRA-K) prepared in 0.001% DMSO. In the assay, 100 µL of a 2.5% RBC suspension was mixed with 100 µL of the brachydin solution (concentrations in the range of 0.00625–0.2 mg/mL) and incubated for 30 min at 37°C. After centrifugation, the supernatant was collected and transferred to a 96-well culture plate to measure the absorbance of the released hemoglobin at 414 nm using a microplate reader. Negative and positive controls were also included in the assay, where RBCs were treated with 0.15 M NaCl and Triton X-100, respectively. This assay was approved by the Ethics Committee on Animal Experimentation of UFMA, Brazil, under protocol number 23115.004153/2022–58.

2.4. Brachydins Structures and ADMET Properties

The chemical structure of each of the brachydins (BRA-G, BRA-I, BRA-J, BRA-K) was drawn using the ChemDraw® JS software version 19.0.0. Representations of their three-dimensional (3D) structures were predicted using the simplified molecular-input line-entry system (SMILES) in mol2 format. The molecular structure of the brachydins was geometrically optimized by means of classical force field calculations using the Avogadro® freeware set up at MMFF94, using the algorithm steepest descent [27]. The PreADMET software was used to assess the ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of brachydins, following the methodology described in previous studies [28, 29].

2.5. Molecular Docking Analysis of Brachydins with GST from *Rhipicephalus microplus*

The *R. microplus* GST 3D structure model was created and validated as previously described [4]. Briefly, the GST amino-acid sequence of *R. microplus* (GenBank number AAL99403.1) was used as a query on Phyre 2 server [30], with normal modelling mode, to create the tick GST structure model. The model was validated using the PROCHECK 3.0 server [31].

Molecular docking studies were performed to assess the potential binding pose and affinity of brachydins (BRA-G, BRA-I, BRA-J, BRA-K) on the H-site of the tick GST enzyme structure model using Molegro Virtual Docker 6.0 (MVD). The docking protocol parameters were: plants score as score function and the iterated simplex (Ant Colony Op-

timization) as the search algorithm. Molecular docking was carried out inside a virtual docking sphere of 15 Å radius and the following center coordinates X: 6.06; Y: 3.61; Z: 28.00 Å. This enabled to obtain MolDock scores as a measure of affinity. The more negative the number, the better the binding. The best pose of each brachydin bound to the GST was visualized and subjected to analysis utilizing the PyMOL Molecular Graphics System v1.3 software (http://www.pymol.org/). Residues within 3.5 Å of brachydins (taken from each best pose) were assessed.

2.6. Statistical Analysis

Statistical analysis was conducted on the mean values obtained from the enzymatic inhibition tests using ANOVA, followed by Tukey's test at a significance level of $p < 0.05$, using GraphPad Prism 8.0.2 software, and the significance of each concentration in the tests was established based on the non-overlapping confidence intervals [32].

3. RESULTS

The HPLC-UV/MS hydroethanolic extract from *F. platyphylla* resulted in 12 peaks. Peaks 7, 9, 10 and 11 were taken, and the compounds they contained were identified as dimeric flavonoid brachydins BRA-G, BRA-I, BRA-J, and BRA-K (Fig. **1**). The structures of these brachydins are shown in Supplementary Fig. (**S1**).

The inhibitory effect of brachydins on the r*Rm*GST activity was determined at fixed concentrations of CDNB (3 mM) and GSH (3 mM). *Rhipicephalus microplus* GST was shown to be inhibited by BRA-G, BRA-I, BRA-J, BRA-K with IC_{50} values of 0.075, 0.079, 0.075, and 0.058 mg/mL, respectively (Table **1**). Furthermore, a concentration of the brachydins up to 0.2 mg/mL resulted in minimal hemolysis of animal red blood cells (Table **1**).

The MolDock scores of the docking simulations were -142.537, -126.831, -108.571, and -123.041 for BRA-G, BRA-I, BRA-J, and BRA-K, respectively. The residues within 3.5 Å of brachydins, probably involved in GST-brachydins interactions, are highlighted in Fig. (**2**).

The physicochemical characteristics and predicted ADMET properties of brachydins are shown in Supplementary Table **1**. BRA-G, BRA-I, BRA-J, and BRA-K present molecular weights of 700.220, 714.230, 684.220 and 538.200 g/mol, respectively. BRA-G, BRA-I and BRA-J have parti-

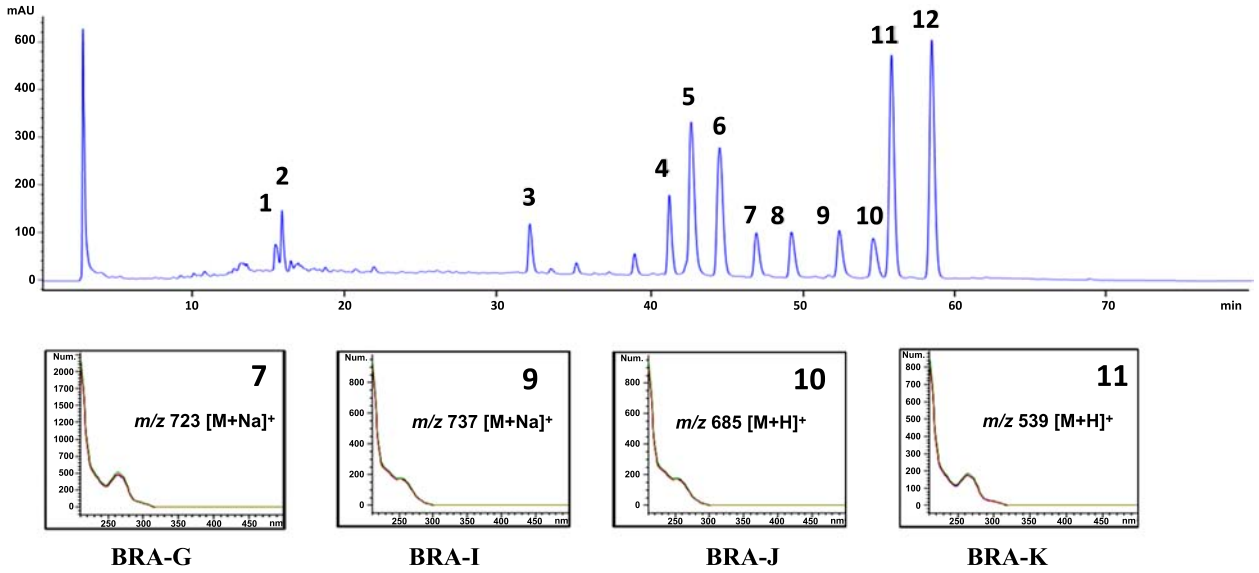

Fig. (1). HPLC-UV/MS analysis of the hydroethanolic extract from *Fridericia platyphylla* at 254 nm and identification of brachydins G, I, J, and K, adapted from Da Rocha *et al*. [22]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Inhibition of GST of *Rhipicephalus microplus* activity by brachydins and the hemolytic activity of the molecules on bovine erythrocytes.

Brachydins	IC_{50} (mg/mL)	CI 95%	R^2	Hemolysis (%)*
BRA-G	0.075[a]	0.065 - 0.088	0.90	0.99 ± 0.26
BRA-I	0.079[a]	0.068 - 0.091	0.93	1.90 ± 0.13
BRA-J	0.075[a]	0.064 - 0.088	0.91	1.08 ± 0.37
BRA-K	0.058[a]	0.049 - 0.068	0.90	1.06 ± 0.65

Note: IC_{50}: Concentration (mg/mL) resulting in 50% of inhibition; CI: 95% confidence interval. The same superscript letter in the same column indicates that the values do not differ significantly at $p < 0.05$. R^2: Regression Correlation Coefficient. *The values given for the percentage of hemolysis were obtained at the maximal BRA concentration tested, *i.e.*, 0.2 mg/mL.

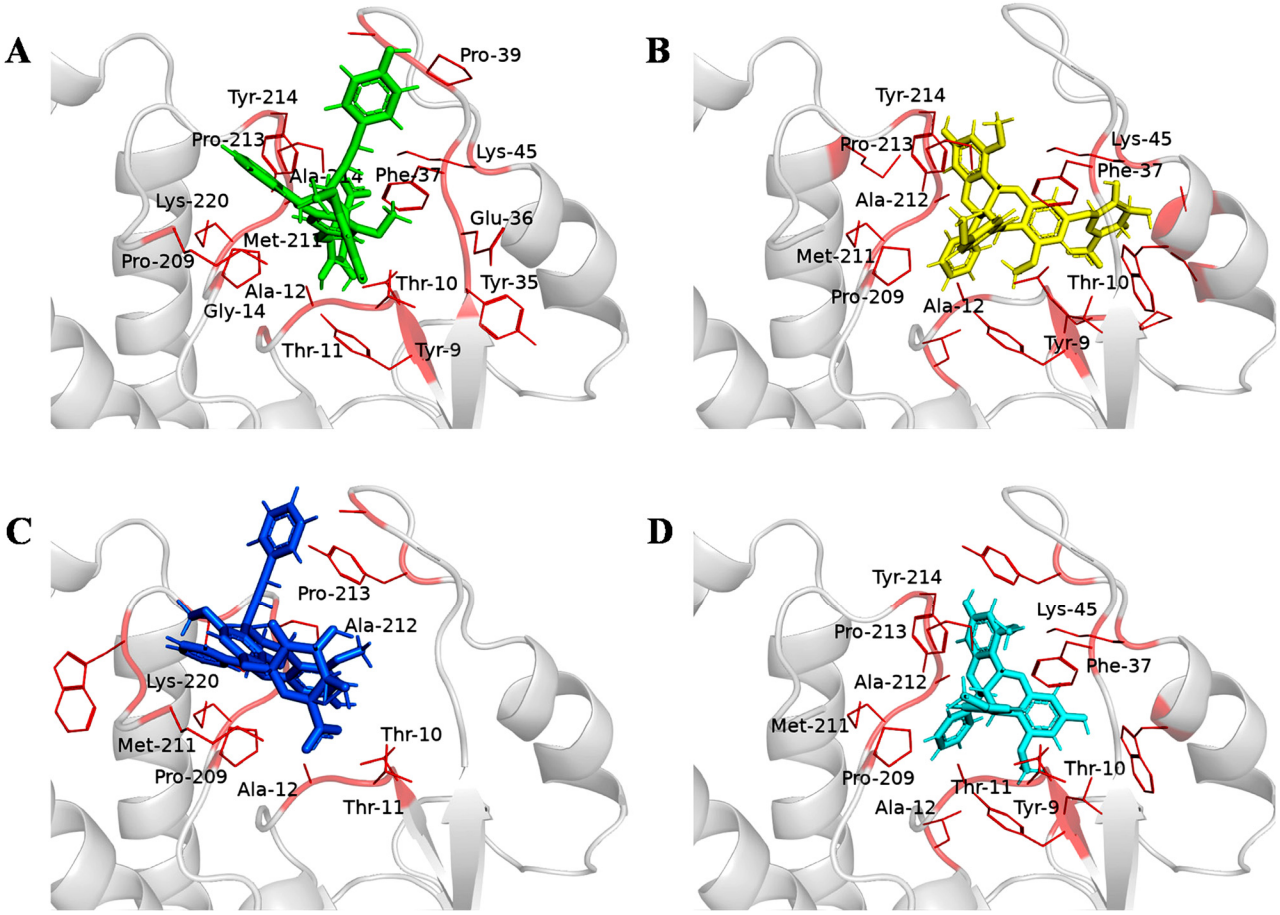

Fig. (2). Cartoon representation of GST of *Rhipicephalus microplus* in complex with **A**) BRA-G; **B**) BRA-I; **C**) BRA-J; and **D**) BRA-K. The ligands are shown as sticks. In red, GST residues within 3.5 Å of brachydins. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

tion coefficient (LogP) values below 5.0. The BRA-G, BRA-I, BRA-J, and BRA-K are predicted to have high human intestinal absorption (87.927%, 92.470%, 92.411%, and 96.410%, respectively). BRA-G showed positive AMES mutagenicity. Moreover, BRA-G, BRA-I and BRA-J may enter the brain since the predicted values for their distribution across the blood-brain barrier (BBB) were 0.0413806, 0.0208044, and 0.0238708, respectively.

4. DISCUSSION

Synthetic acaricides have become commonly employed in veterinary and human medicine for the control of parasitic diseases. However, the emergence of resistance has underscored the need for alternative approaches [33]. In response, there is an increasing interest in the exploration of novel bioactive compounds, such as flavonoids possessing enhanced potency and selectivity toward tick targets, thus presenting prospective drugs to control this parasite. Computational and *in vitro* methods facilitate the discovery of novel compounds capable of binding to molecular targets within parasites [34]. Accordingly, this study provides both *in silico* and *in vitro* evidence of the inhibition of *R. microplus* GST by the flavonoid brachydins BRA-G, BRA-I, BRA-J, and BRA-K (Table **1** and Fig. **2**). Dimeric flavonoids exhibit a wide range of bioactivities, including antiproliferative and antiprotozoal properties and it has been suggested that the chalcone elements in such flavonoid structures contribute considerably to their pharmacological potential [16, 17, 35].

Although flavonoids have been demonstrated to modulate GST activity [36], to the best of our knowledge, this is the first study on GST inhibition by brachydins. Furthermore, in ticks, the well-documented role of GST in metabolizing endo- and xenobiotic compounds, supported by elevated transcription rates of the GST gene and increased enzyme activity when exposed to these compounds, positions GSTs as promising targets for the development of novel acaricidal drugs [5]. Additionally, this study shows that BRA-G, BRA-I, BRA-J, and BRA-K, up to 0.2 mg/mL, caused only limited RBC hemolysis, with values below 2% (Table **1**). Limited research has explored the toxicity of brachydins on mammalian cells. For example, brachydins E and F, from *F. platyphylla* roots, exhibited cytotoxicity in non-tumoral keratinocyte cells, with IC_{50} values of 50.5 and 59.9 µg/mL, respectively [35]. Additionally, brachydins A, B, and C, isolated from *F. platyphylla*, did not demonstrate toxicity to peritoneal macrophages at a concentration of 20 µM [17]. Toxicity assessments are important in the early stages of drug development, allowing the evaluation of a substance's safety [37, 38].

Various synthetic and naturally occurring compounds have been reported as GST inhibitors, and their activities generally hinge on specific structural characteristics [39]. The potential of brachydins G, I, J, and K to inhibit *R. microplus* GST was also evaluated *in silico* (Fig. **2**). Brachydins showed high binding energies (MolDock scores <-100) towards GST from *R. microplus*. MolDock has a very high docking accuracy when it comes to identifying ligand binding modes. The interaction score represents the total energy for the interaction between the ligand and the protein, so the lower the value, the better the interaction [40, 41]. In this study, we assessed GST residues within a proximity of 3.5 Å to brachydins, suggested as an ideal interaction distance [42, 43]. The residues of GST interacting with brachydins are located within the H site, which is a hydrophobic area. Given the significance of both the G and H sites for GST activity, it is suggested that brachydins may disrupt enzymatic activity through their interaction with crucial H-site residues. Residues Thr10, Ala12, Pro209, Met211, and Pro213 in *R. microplus* GST may be key residues potentially involved in the interaction, as they are all within 3.5 Å of each brachydin (Fig. **2**). Indeed, the interaction between the acaricide cyflumetofen and a GST from a resistant strain of *Tetranychus urticae*, namely GST TuGSTd05, which is considered a key candidate in conferring resistance, is facilitated by hydrophobic interactions involving residues such as Ala12 [44].

The physicochemical parameters and the predicted ADMET properties of brachydins were analyzed by the PreADMET tool (Supplementary Table **S1**). BRA-G, BRA-I, BRA-J, and BRA-K are predicted to exhibit high absorption, a critical factor to consider when contemplating the oral administration of drugs as potential therapeutic agents [45, 46].

Despite the well-established interaction of some flavonoids with membranes [47], limited data are available on the penetration of brachydins through the tick cuticle and membranes. According to Lipinski's Rule of 5, a compound is considered drug-like if it complies with the following criteria: a molecular weight less than 500 Da, a partition coefficient (logP) below 5, and having maximally five hydrogen bond donors and ten hydrogen bond acceptors [48]. In this study, brachydins violated at least two of Lipinski's Rule parameters (Supplementary Table **S1**). However, many natural products that violate Lipinski's Rule criteria do traverse cell membranes [49-51], a notable example being suramin, known for its antiparasitic activity against *Trypanosoma brucei* [52]. This drug enters the parasite by binding to a surface glycoprotein that mediates its internalization [52]. Additionally, orally active therapeutics of various categories that are not compliant with Lipinski's Rule serve as substrates for biological transporters [48]. This keeps open the possibility that also brachydins, which are unlikely to diffuse passively through the lipid bilayer, may enter cells by such a mechanism. This notion is supported by the fact that many physicochemical characteristics of BRA-G, BRA-I, and BRA-J are similar to BRA-E and BRA-F, which have previously been demonstrated to exert antiproliferative activity on different tumor cell lines, rendering likely their import into these cells [35].

BRA-G, BRA-I, BRA-J, and BRA-K, at 0.2 mg/mL, appeared not effective in preliminary *R. microplus* larval immersion tests. However, BRA-G significantly increased the effect of cypermethrin in such assays (data not shown). This observation suggests that, by inhibiting the GST of *R. microplus*, BRA-G interferes with the detoxification process of cypermethrin within the tick and potentiates the larvicidal effect of this pyrethroid.

It is well-established that prolonged and improper use of acaricides can enhance tolerance and resistance in ticks, leading to resistance evolution in various species [11]. Bioactive metabolites provide a promising alternative for controlling ticks, even those resistant to conventional acaricides [53]. Targeting key tick enzymes like GST represents an innovative approach with the potential for new drug development. Based on the findings of this study, flavonoids BRA-G, BRA-I, BRA-J, and BRA-K may function as inhibitors of the GST of *R. microplus*. Expanding research into GST inhibition by brachydins and towards modifying the structures and/or elaborate formulations with these compounds to increase their efficacy may pave the way for their possible application as acaricides, contributing to the development of innovative anti-tick drugs.

CONCLUSION

In conclusion, this study underscores the potential of brachydins, particularly BRA-G, BRA-I, BRA-J, and BRA-K, as inhibitors of *R. microplus* GST. These findings hold promise for the development of novel strategies in combating acaricide-resistant cattle ectoparasites, offering a potential avenue to mitigate economic losses in the livestock industry. Further research and field trials are warranted to explore the practical application of brachydins for tick control.

AUTHORS' CONTRIBUTIONS

WASB: Investigation, Methodology, Formal analysis, Writing – original draft. CPT: Methodology, Formal analysis, Investigation. VASL: Methodology, Formal analysis, Investigation. CQR: Resources, Investigation, Writing – review & editing. ISVJ: Resources, Investigation, Writing – review & editing. PAMM: Conceptualization, Validation, Data curation, Formal analysis, Writing – review & editing. LMCJ: Resources, Supervision, Writing – review & editing, Validation. AMSS: Conceptualization, Resources, Funding acquisition, Writing – review & editing, Validation, Project administration.

LIST OF ABBREVIATIONS

BRAs	=	Brachydins
GST	=	Glutathione S-transferase
MPLC	=	Medium-pressure liquid chromatography

ETHICAL STATEMENT

This assay was approved by the Ethics Committee on Animal Experimentation of UFMA, Brazil, under protocol number 23115.004153/2022-58.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

FUNDING

This study was supported in part by Maranhão State Research Foundation (FAPEMA) - INFRA 03170/18, UNIVERSAL 00869/22 and FAPEMA IECT Biotechnology/Financier of Studies and Projects (FINEP) process 2677/17. It was also financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Finance Code 001).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This study was supported in part by Maranhão State Research Foundation (FAPEMA) - INFRA 03170/18, UNIVERSAL 00869/22 and FAPEMA IECT Biotechnology/ Financier of Studies and Projects (FINEP) process 2677/17. It was also financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Finance Code 001).

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Grisi, L.; Leite, R.C.; Martins, J.R.S.; Barros, A.T.M.; Andreotti, R.; Cançado, P.H.D.; León, A.A.P.; Pereira, J.B.; Villela, H.S. Reassessment of the potential economic impact of cattle parasites in Brazil. *Rev. Bras. Parasitol. Vet.,* **2014**, *23*(2), 150-156. http://dx.doi.org/10.1590/S1984-29612014042 PMID: 25054492

[2] Agwunobi, D.O.; Yu, Z.; Liu, J. A retrospective review on ixodid tick resistance against synthetic acaricides: implications and perspectives for future resistance prevention and mitigation. *Pestic. Biochem. Physiol.,* **2021**, *173*(1), 104776. http://dx.doi.org/10.1016/j.pestbp.2021.104776 PMID: 33771255

[3] Waldman, J.; Klafke, G.M.; Tirloni, L.; Logullo, C.; da Silva Vaz, I., Jr Putative target sites in synganglion for novel ixodid tick control strategies. *Ticks Tick Borne Dis.,* **2023**, *14*(3), 102123. http://dx.doi.org/10.1016/j.ttbdis.2023.102123 PMID: 36716581

[4] Bezerra, W.A.S.; Tavares, C.P.; Rocha, C.Q.; Vaz Junior, I.S.; Michels, P.A.M.; Costa Junior, L.M.; Soares, A.M.S. Anonaine from Annona crassiflora inhibits glutathione S-transferase and improves cypermethrin activity on Rhipicephalus (Boophilus) microplus (Canestrini, 1887). *Exp. Parasitol.,* **2022**, *243*, 108398. http://dx.doi.org/10.1016/j.exppara.2022.108398 PMID: 36228702

[5] Ozelame, K.P.C.; Mattia, M.M.C.; Dedavid e Silva, L.A.; Randall, L.M.; Corvo, I.; Saporiti, T.; Seixas, A.; da Silva Vaz, I., Jr; Alvarez, G. Novel tick glutathione transferase inhibitors as promising acaricidal compounds. *Ticks Tick Borne Dis.,* **2022**, *13*(5), 101970. http://dx.doi.org/10.1016/j.ttbdis.2022.101970 PMID: 35662066

[6] Thakur, A.; Patwa, J.; Sharma, A.; Flora, S.J.S. Synthesis, molecular docking, BSA, and *in vitro* reactivation study of imidazopyridine oximes against paraoxon inhibited acetylcholinesterase. *Med. Chem.,* **2022**, *18*(2), 273-287. http://dx.doi.org/10.2174/1573406417666210208223240 PMID: 33563155

[7] Otarigho, B.; Falade, M.O. Natural Perylenequinone Compounds as Potent Inhibitors of *Schistosoma mansoni* Glutathione S-Transferase. *Life,* **2023**, *13*(10), 1957. http://dx.doi.org/10.3390/life13101957 PMID: 37895339

[8] Turkan, F.; Harbi Calimli, M.; Akgun, A.; Gulbagca, F.; Sen, F. Toxicological effects of some antiparasitic drugs on equine liver glutathione S-Transferase enzyme activity. *J. Pharm. Biomed. Anal.,* **2020**, *180*, 113048. http://dx.doi.org/10.1016/j.jpba.2019.113048 PMID: 31887670

[9] Costa-Júnior, L.M.; Miller, R.J.; Alves, P.B.; Blank, A.F.; Li, A.Y.; Pérez de León, A.A. Acaricidal efficacies of *Lippia gracilis* essential oil and its phytochemicals against organophosphate-resistant and susceptible strains of *Rhipicephalus (Boophilus) microplus. Vet. Parasitol.,* **2016**, *228*(1), 60-64. http://dx.doi.org/10.1016/j.vetpar.2016.05.028 PMID: 27692332

[10] Quadros, D.G.; Johnson, T.L.; Whitney, T.R.; Oliver, J.D.; Oliva Chávez, A.S. Plant-derived natural compounds for tick pest control in livestock and wildlife: Pragmatism or Utopia? *Insects,* **2020**, *11*(8), 490. http://dx.doi.org/10.3390/insects11080490 PMID: 32752256

[11] Selles, S.M.A.; Kouidri, M.; González, M.G.; González, J.; Sánchez, M.; González-Coloma, A.; Sanchis, J.; Elhachimi, L.; Olmeda, A.S.; Tercero, J.M.; Valcárcel, F. Acaricidal and repellent effects of essential oils against ticks: a review. *Pathogens,* **2021**, *10*(11), 1379. http://dx.doi.org/10.3390/pathogens10111379 PMID: 34832535

[12] El Haddad, D.; Bitam, I.; Bouchenak, O.; Toubal, S.; Yahiaoui, K.; Arab, K.; Boumaza, S. Acaricidal activity of flavonoids extract of *Borago officinalis* L. (Boraginaceae) against brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806). *Trop. Biomed.,* **2018**, *35*(2), 383-391. PMID: 33601812

[13] Cen-Pacheco, F.; Ortiz-Celiseo, A.; Peniche-Cardeña, A.; Bravo-Ruiz, O.; López-Fentanes, F.C.; Valerio-Alfaro, G.; Fernández, J.J. Studies on the bioactive flavonoids isolated from *Azadirachta indica*. *Nat. Prod. Res.,* **2020**, *34*(24), 3483-3491. http://dx.doi.org/10.1080/14786419.2019.1579808 PMID: 30835540

[14] Hamed, R.R.; Maharem, T.M.; Guneidy, R.A.; Emam, M.A.; Abdel Karim, G.S.A. Purification of fat body glutathione *S* ‑transferase from the desert locust *Schistocerca gregaria* : investigation of flavonoid inhibitory effects on enzyme activity. *Physiol. Entomol.,* **2019**, *44*(3-4), 187-199. http://dx.doi.org/10.1111/phen.12289

[15] Inaba, K.; Ebihara, K.; Senda, M.; Yoshino, R.; Sakuma, C.; Koiwai, K.; Takaya, D.; Watanabe, C.; Watanabe, A.; Kawashima, Y.; Fukuzawa, K.; Imamura, R.; Kojima, H.; Okabe, T.; Uemura, N.; Kasai, S.; Kanuka, H.; Nishimura, T.; Watanabe, K.; Inoue, H.; Fujikawa, Y.; Honma, T.; Hirokawa, T.; Senda, T.; Niwa, R. Molecular action of larvicidal flavonoids on ecdysteroidogenic glutathione S-transferase Noppera-bo in *Aedes aegypti. BMC Biol.,* **2022**, *20*(1), 43. http://dx.doi.org/10.1186/s12915-022-01233-2 PMID: 35172816

[16] da Rocha, C.Q.; Queiroz, E.F.; Meira, C.S.; Moreira, D.R.M.; Soares, M.B.P.; Marcourt, L.; Vilegas, W.; Wolfender, J.L. Dimeric flavonoids from *Arrabidaea brachypoda* and assessment of their anti-*Trypanosoma cruzi* activity. *J. Nat. Prod.,* **2014**, *77*(6), 1345-1350. http://dx.doi.org/10.1021/np401060j PMID: 24871307

[17] Rocha, V.; Quintino da Rocha, C.; Ferreira Queiroz, E.; Marcourt, L.; Vilegas, W.; Grimaldi, G.; Furrer, P.; Allémann, É.; Wolfender, J.L.; Soares, M. Antileishmanial activity of dimeric flavonoids isolated from *Arrabidaea brachypoda. Molecules,* **2018**, *24*(1), 1-13. http://dx.doi.org/10.3390/molecules24010001 PMID: 30577423

[18] Saramago, L.; Gomes, H.; Aguilera, E.; Cerecetto, H.; González, M.; Cabrera, M.; Alzugaray, M.; da Silva Vaz Junior, I.; Nunes da Fonseca, R.; Aguirre-López, B.; Cabrera, N.; Pérez-Montfort, R.; Merlino, A.; Moraes, J.; Álvarez, G. Novel and selective *Rhipicephalus microplus* triosephosphate isomerase inhibitors with acaricidal activity. *Vet. Sci.,* **2018**, *5*(3), 74. http://dx.doi.org/10.3390/vetsci5030074 PMID: 30142944

[19] Shaker, B.; Ahmad, S.; Lee, J.; Jung, C.; Na, D. *In silico* methods and tools for drug discovery. *Comput. Biol. Med.,* **2021**, *137*, 104851.

http://dx.doi.org/10.1016/j.compbiomed.2021.104851 PMID: 34520990

[20] Bitar, R.; Parker, M.; Walker, J. *Drug discovery. Translational interventional radiology*; Elsevier, **2023**, pp. 51-54. http://dx.doi.org/10.1016/B978-0-12-823026-8.00029-8

[21] Adibi, H.; Khodarahmi, R.; Ezati, M.; Ghavamipour, F.; Khosravi, N.; Sajedi, R.H.; Chalabi, M.; Farokhi, A. Synthesis and potential antidiabetic properties of curcumin-based derivatives: an *in vitro* and *in silico* study of α-glucosidase and α-amylase inhibition. *Med. Chem.,* **2023**, *19*(1), 99-117. http://dx.doi.org/10.2174/1573406418666220509101854 PMID: 35579152

[22] da Rocha, C.Q.; de-Faria, F.M.; Marcourt, L.; Ebrahimi, S.N.; Kitano, B.T.; Ghilardi, A.F.; Luiz Ferreira, A.; de Almeida, A.C.A.; Dunder, R.J.; Souza-Brito, A.R.M.; Hamburger, M.; Vilegas, W.; Queiroz, E.F.; Wolfender, J.L. Gastroprotective effects of hydroethanolic root extract of *Arrabidaea brachypoda*: Evidences of cytoprotection and isolation of unusual glycosylated polyphenols. *Phytochemistry,* **2017**, *135*, 93-105. http://dx.doi.org/10.1016/j.phytochem.2016.12.002 PMID: 28010885

[23] da Silva Vaz, I., Jr; Torino Lermen, T.; Michelon, A.; Sanchez Ferreira, C.A.; Joaquim de Freitas, D.R.; Termignoni, C.; Masuda, A. Effect of acaricides on the activity of a *Boophilus microplus* glutathione S-transferase. *Vet. Parasitol.,* **2004**, *119*(2-3), 237-245. http://dx.doi.org/10.1016/j.vetpar.2003.11.004 PMID: 14746982

[24] Ndawula, C., Jr; Sabadin, G.A.; Parizi, L.F.; da Silva Vaz, I., Jr Constituting a glutathione S-transferase-cocktail vaccine against tick infestation. *Vaccine,* **2019**, *37*(14), 1918-1927. http://dx.doi.org/10.1016/j.vaccine.2019.02.039 PMID: 30824358

[25] Habig, W.H.; Pabst, M.J.; Fleischner, G.; Gatmaitan, Z.; Arias, I.M.; Jakoby, W.B. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. *Proc. Natl. Acad. Sci. USA,* **1974**, *71*(10), 3879-3882. http://dx.doi.org/10.1073/pnas.71.10.3879 PMID: 4139704

[26] Choi, J.; Reipa, V.; Hitchins, V.M.; Goering, P.L.; Malinauskas, R.A. Physicochemical characterization and *in vitro* hemolysis evaluation of silver nanoparticles. *Toxicol. Sci.,* **2011**, *123*(1), 133-143. http://dx.doi.org/10.1093/toxsci/kfr149 PMID: 21652737

[27] Hanwell, M.D.; Curtis, D.E.; Lonie, D.C.; Vandermeersch, T.; Zurek, E.; Hutchison, G.R. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *J. Cheminform.,* **2012**, *4*(1), 17. http://dx.doi.org/10.1186/1758-2946-4-17 PMID: 22889332

[28] Wadapurkar, R.M.; Shilpa, M.D.; Katti, A.K.S.; Sulochana, M.B. *In silico* drug design for *Staphylococcus aureus* and development of host-pathogen interaction network. *Informatics in Medicine Unlocked,* **2018**, *10*, 58-70. http://dx.doi.org/10.1016/j.imu.2017.11.002

[29] Tong, J.B.; Luo, D.; Bian, S.; Zhang, X. Structural investigation of tetrahydropteridin analogues as selective PLK1 inhibitors for treating cancer through combined QSAR techniques, molecular docking, and molecular dynamics simulations. *J. Mol. Liq.,* **2021**, *335*, 116235. http://dx.doi.org/10.1016/j.molliq.2021.116235

[30] Kelley, L.A.; Mezulis, S.; Yates, C.M.; Wass, M.N.; Sternberg, M.J.E. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.,* **2015**, *10*(6), 845-858. http://dx.doi.org/10.1038/nprot.2015.053 PMID: 25950237

[31] Laskowski, R.A.; MacArthur, M.W.; Moss, D.S.; Thornton, J.M. PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Cryst.,* **1993**, *26*(2), 283-291. http://dx.doi.org/10.1107/S0021889892009944

[32] Roditakis, E.; Roditakis, N.E.; Tsagkarakou, A. Insecticide resistance in *Bemisia tabaci* (Homoptera: Aleyrodidae) populations from Crete. *Pest Manag. Sci.,* **2005**, *61*(6), 577-582. http://dx.doi.org/10.1002/ps.1029 PMID: 15712366

[33] Obaid, M.K.; Islam, N.; Alouffi, A.; Khan, A.Z.; da Silva Vaz, I., Jr; Tanaka, T.; Ali, A. Acaricides resistance in ticks: selection, diagnosis, mechanisms, and mitigation. *Front. Cell. Infect. Microbiol.,* **2022**, *12*, 941831. http://dx.doi.org/10.3389/fcimb.2022.941831 PMID: 35873149

[34] Malak, N.; Alotaibi, B.S.; Khan, A.; Khan, A.; Ullah, S.; Nasreen, N.; Niaz, S.; Chen, C.C. Density functional theory calculations and molecular docking analyses of flavonoids for their possible application against the acetylcholinesterase and triose-phosphate isomerase proteins of *Rhipicephalus microplus. Molecules,* **2023**, *28*(8), 3606. http://dx.doi.org/10.3390/molecules28083606 PMID: 37110838

[35] de Lima, C.A.; Cubero, M.C.Z.; Franco, Y.E.M.; Rodrigues, C.D.P.; do Nascimento, J.R.; Vendramini-Costa, D.B.; Sciani, J.M.; da Rocha, C.Q.; Longato, G.B. Antiproliferative activity of two unusual dimeric flavonoids, brachydin E and brachydin F, isolated from *Fridericia platyphylla* (Cham.) LG Lohmann: *in vitro* and molecular docking evaluation. *BioMed Res. Int.,* **2022**, *2022*, 1-12. http://dx.doi.org/10.1155/2022/3319203 PMID: 35187163

[36] Boušová, I.; Skálová, L. Inhibition and induction of glutathione S-transferases by flavonoids: possible pharmacological and toxicological consequences. *Drug Metab. Rev.,* **2012**, *44*(4), 267-286. http://dx.doi.org/10.3109/03602532.2012.713969 PMID: 22998389

[37] Makris, S.L.; Kim, J.H.; Ellis, A.; Faber, W.; Harrouk, W.; Lewis, J.M.; Paule, M.G.; Seed, J.; Tassinari, M.; Tyl, R. Current and future needs for developmental toxicity testing. *Birth Defects Res. B Dev. Reprod. Toxicol.,* **2011**, *92*(5), 384-394. http://dx.doi.org/10.1002/bdrb.20335 PMID: 21922641

[38] Arome, D.; Chinedu, E. The importance of toxicity testing. *Journal of Pharmaceutical BioSciences.,* **2013**, *4*, 146-148.

[39] Ata, A.; Udenigwe, C. The discovery and application of inhibitors of glutathione S-transferase as therapeutic agents-a review. *Curr. Bioact. Compd.,* **2008**, *4*(1), 41-50. http://dx.doi.org/10.2174/157340708784533384

[40] Thomsen, R.; Christensen, M.H. MolDock: a new technique for high-accuracy molecular docking. *J. Med. Chem.,* **2006**, *49*(11), 3315-3321. http://dx.doi.org/10.1021/jm051197e PMID: 16722650

[41] Xavier, M.M.; Heck, G.S.; de Avila, M.B.; Levin, N.M.B.; Pintro, V.O.; Carvalho, N.L.; de Azevedo, W.F. SAnDReS a computational tool for statistical analysis of docking results and development of scoring functions. *Comb. Chem. High Throughput Screen.,* **2016**, *19*(10), 801-812. http://dx.doi.org/10.2174/1386207319666160927111347 PMID: 27686428

[42] Liu, Z.; Wang, G.; Li, Z.; Wang, R. Geometrical preferences of the hydrogen bonds on protein−ligand binding interface derived from statistical surveys and quantum mechanics calculations. *J. Chem. Theory Comput.,* **2008**, *4*(11), 1959-1973. http://dx.doi.org/10.1021/ct800267x PMID: 26620338

[43] Bianchi, V.; Gherardini, P.F.; Helmer-Citterich, M.; Ausiello, G. Identification of binding pockets in protein structures using a knowledge-based potential derived from local structural similarities. *BMC Bioinformatics,* **2012**, *13*(S4)(Suppl. 4), S17. http://dx.doi.org/10.1186/1471-2105-13-S4-S17 PMID: 22536963

[44] Pavlidi, N.; Khalighi, M.; Myridakis, A.; Dermauw, W.; Wybouw, N.; Tsakireli, D.; Stephanou, E.G.; Labrou, N.E.; Vontas, J.; Van Leeuwen, T. A glutathione-S-transferase (TuGSTd05) associated with acaricide resistance in *Tetranychus urticae* directly metabolizes the complex II inhibitor cyflumetofen. *Insect Biochem. Mol. Biol.,* **2017**, *80*, 101-115. http://dx.doi.org/10.1016/j.ibmb.2016.12.003 PMID: 27932274

[45] Hou, T.; Wang, J.; Li, Y. ADME evaluation in drug discovery. 8. The prediction of human intestinal absorption by a support vector machine. *J. Chem. Inf. Model.,* **2007**, *47*(6), 2408-2415. http://dx.doi.org/10.1021/ci7002076 PMID: 17929911

[46] Hou, T.; Wang, J.; Zhang, W.; Xu, X. ADME evaluation in drug discovery. 7. Prediction of oral absorption by correlation and classification. *J. Chem. Inf. Model.,* **2007**, *47*(1), 208-218. http://dx.doi.org/10.1021/ci600343x PMID: 17238266

[47] Erlejman, A.G.; Verstraeten, S.V.; Fraga, C.G.; Oteiza, P.I. The interaction of flavonoids with membranes: potential determinant of flavonoid antioxidant effects. *Free Radic. Res.,* **2004**, *38*(12), 1311-1320. http://dx.doi.org/10.1080/10715760400016105 PMID: 15763955

[48] Lipinski, C.A.; Lombardo, F.; Dominy, B.W.; Feeney, P.J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings 1PII of original article: S0169-409X(96)00423-1. The article was originally published in Advanced Drug Delivery Reviews 23 (1997) 3–25. 1. *Adv. Drug Deliv. Rev.,* **2001**, *46*(1-3), 3-26. http://dx.doi.org/10.1016/S0169-409X(00)00129-0 PMID: 11259830

[49] Leeson, P.D.; Davis, A.M. Time-related differences in the physical property profiles of oral drugs. *J. Med. Chem.,* **2004**, *47*(25), 6338-6348. http://dx.doi.org/10.1021/jm049717d PMID: 15566303

[50] O'Shea, R.; Moser, H.E. Physicochemical properties of antibacterial compounds: implications for drug discovery. *J. Med. Chem.,* **2008**, *51*(10), 2871-2878. http://dx.doi.org/10.1021/jm700967e PMID: 18260614

[51] Abdelmohsen, U.R.; Balasubramanian, S.; Oelschlaeger, T.A.; Grkovic, T.; Pham, N.B.; Quinn, R.J.; Hentschel, U. Potential of marine natural products against drug-resistant fungal, viral, and parasitic infections. *Lancet Infect. Dis.,* **2017**, *17*(2), e30-e41. http://dx.doi.org/10.1016/S1473-3099(16)30323-1 PMID: 27979695

[52] Wiedemar, N; Hauser, DA; Mäser, P 100 years of suramin. *Antimicrobi. agen. chemother.,* **2020**, *64*(3), 1-14. http://dx.doi.org/10.1128/AAC.01168-19

[53] Adenubi, O.T.; Ahmed, A.S.; Fasina, F.O.; McGaw, L.J.; Eloff, J.N.; Naidoo, V. Pesticidal plants as a possible alternative to synthetic acaricides in tick control: A systematic review and meta-analysis. *Ind. Crops Prod.,* **2018**, *123*(1), 779-806. http://dx.doi.org/10.1016/j.indcrop.2018.06.075

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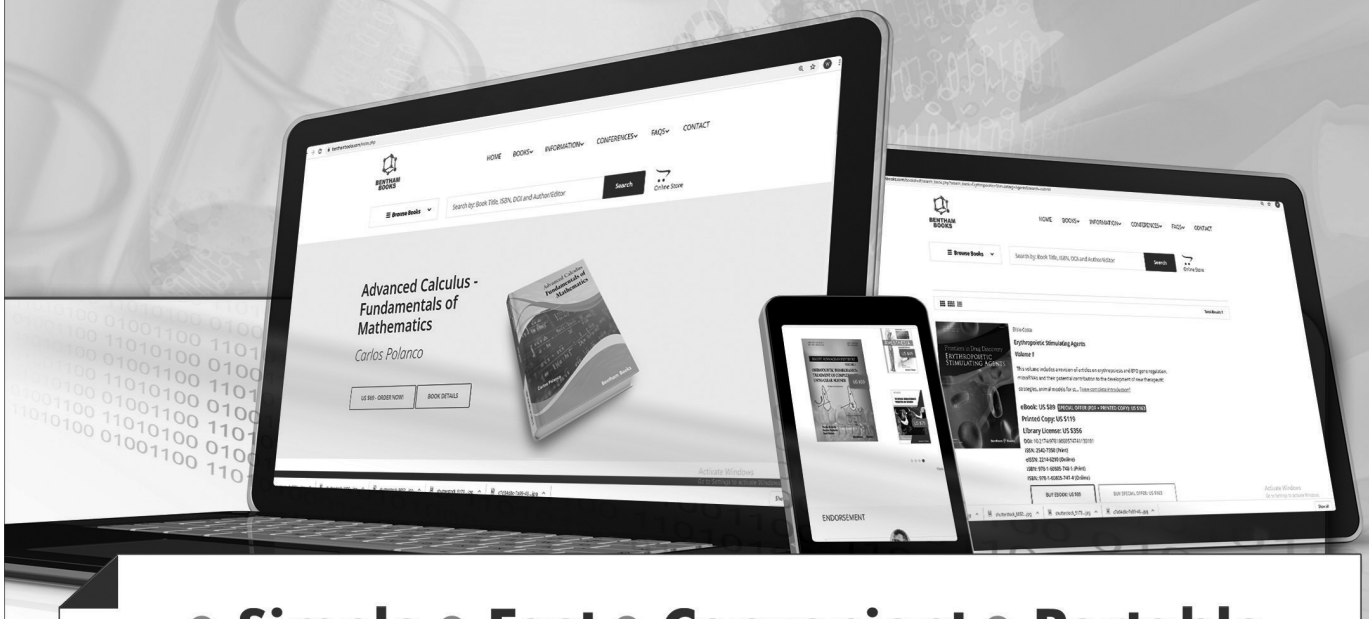

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Experimental Parasitology 243 (2022) 108398

Contents lists available at ScienceDirect

Experimental Parasitology

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

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ARTICLE INFO

Keywords:
Tick
Plant alkaloid
GST inhibition
Pyrethroid

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 (r*Rm*GST) 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*, r*Rm*GST 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 LC_{50} 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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https://doi.org/10.1016/j.exppara.2022.108398
Received 1 June 2022; Received in revised form 26 September 2022; Accepted 29 September 2022
Available online 11 October 2022
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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-Hernándes 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 Avogrado 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 (RSMD) less than 2 Å from the original structure (Yusuf et al., 2008). The re-docking protocol result with the lowest RSMD 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% $NaHCO_3$ solution and eluted with gradients of petroleum ether: CH_2Cl_2, then gradients of CH_2Cl_2: EtOAc, and finally gradients of EtOAc: CH_3OH, 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 CH_2Cl_2: 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 (r*Rm*GST) was expressed and purified as previously described (Ndawula et al., 2019).

Briefly, *Escherichia coli* (Migula 1895) BL21(DE3) was transformed with plasmid and the r*Rm*GST 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 r*Rm*GST was eluted with 50 mM Tris-HCl pH 8.0 containing 10 mM reduced glutathione (GSH). The expression and purification of r*Rm*GST were monitored by SDS–PAGE and western blotting using anti-r*Rm*GST 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 CDNB 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 CDNB, 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, CDNB, 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 LC_{50} (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

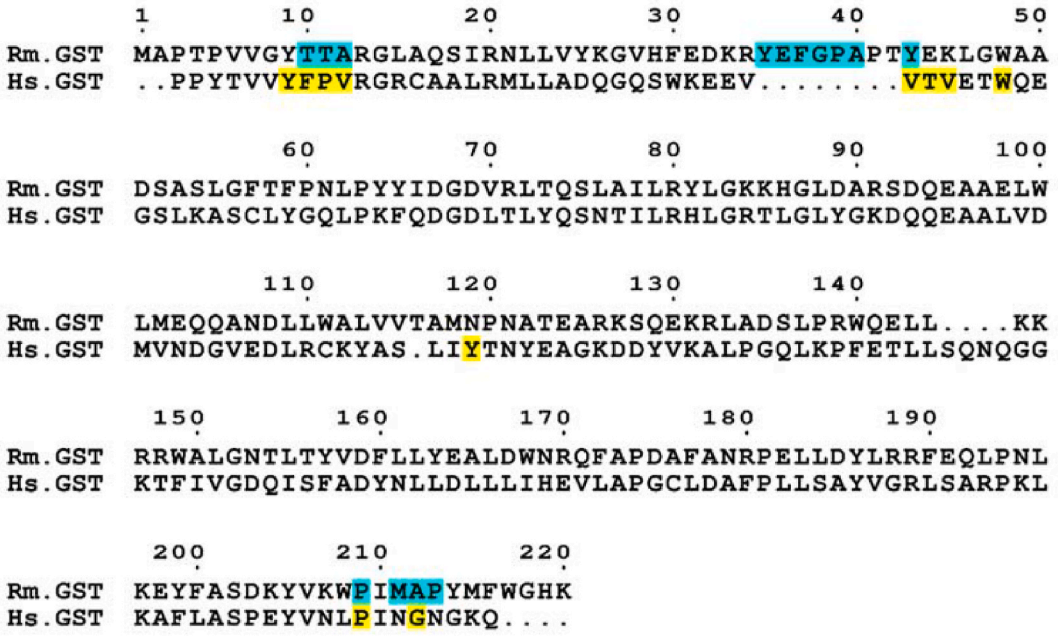

Fig. 1. Protein sequence alignment of the human GST (Hs.GST), (PDB ID: 3IE3-Chain A) and *Rhipicephalus microplus* GST (Rm.GST). Residues of human GST and tick GST interacting with anonaine are highlighted in yellow and blue, respectively.

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.

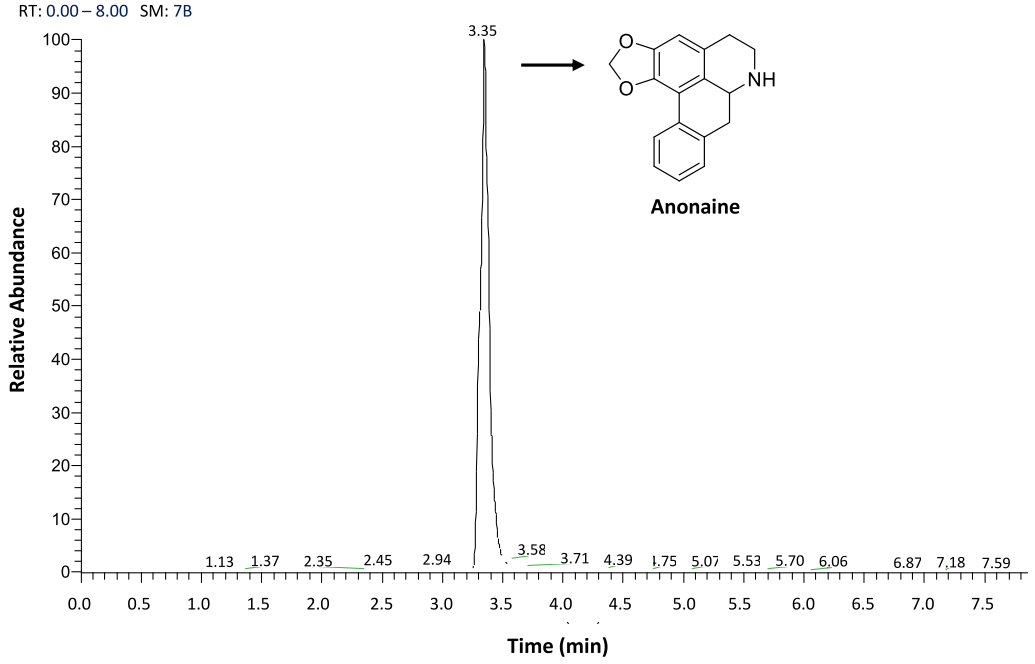

Fig. 2. Chromatogram of total ions of anonaine, isolated from *Annona crassiflora*. Inset: anonaine structure.

A single protein band was observed in SDS-PAGE and Western blot analyses of r*Rm*GST 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 r*Rm*GST 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 LC_{50} 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 ± 8.74% and 14.26 ± 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 RSMD 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.

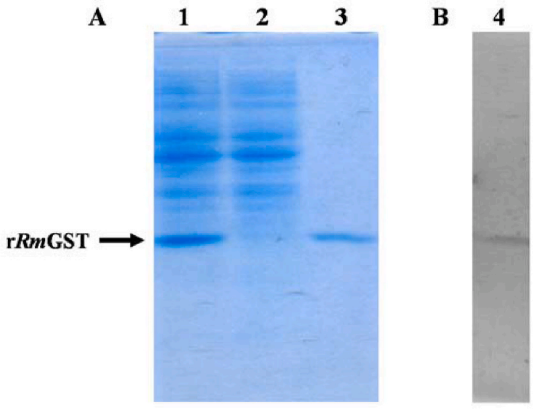

Fig. 3. A) SDS-PAGE (12% gel, with electrophoresis performed under reducing conditions) and B) Western blot of recombinant *R. microplus* GST. 1) Extract of *E. coli* cells expressing r*Rm*GST; 2) Unbound fraction eluted in GSH chromatography; 3) Purified GST (r*Rm*GST); 4) Western blot with anti-GST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

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 r*Rm*GST 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.

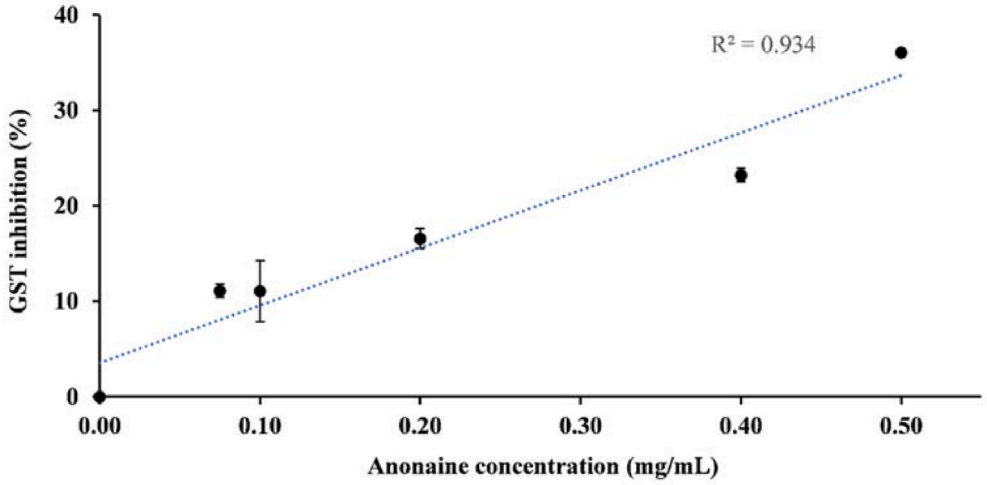

Figura 4. Inhibition curve for the anonaine on r*Rm*GST. Y-axis: percentage of GST inhibition; X-axis: anonaine concentration in mg/mL.

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; LC_{50}: Lethal concentration (mg/mL) for 50% of individuals; CI: 95% confidence interval; R^2: 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 r*Rm*GST 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.

Acknowledgements

This study was supported in part by Maranhão State Research Foundation (FAPEMA) - INFRA 03170/18, UNIVERSAL 00869/22 and FAPEMA IECT Biotechnology/Financier of Studies and Projects (FINEP) process 2677/17. It was also financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Finance Code 001). The authors thank Dr Walter F. de Azevedo Jr. (Pontifical Catholic University of Rio Grande do Sul) for his assistance.

Appendix A. Supplementary data

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

References

Adenubi, O.T., Ahmed, A.S., Fasina, F.O., McGaw, L.J., Eloff, J.N., Naidoo, V., 2018. Pesticidal plants as a possible alternative to synthetic acaricides in tick control: a systematic review and meta-analysis. Ind. Crop. Prod. 123, 779–806.

Ahmad, R., Srivastava, A.K., Walter, R.D., 2008. Purification and biochemical characterization of cytosolic glutathione-S-transferase from filarial worms Setaria cervi. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 151, 237–245.

Alliance, D., 2016. Estudo químico, predições in silico das propriedades ADME/TOX e atividade larvicida do óleo essencial da raiz Philodendron deflexum Poepp. In: Ex Schott sobre Aedes egypti Linneu e Anopheles albirtasis sl, Department of Graduation Graduation Program in Health Sciences - Ppgcs. Federal University Of Amapa, Macapá - Ap. Brazil., pp. 1–53

Alvarez, J.C., 2004. High-throughput docking as a source of novel drug leads. Curr. Opin. Chem. Biol. 8, 365–370.

Ames, B.N., Gurney, E., Miller, J.A., Bartsch, H., 1972. Carcinogens as frameshift mutagens: metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proceedings of the National Academy of Sciences of the U.S.A. 69, 3128–3132.

Azeez, S., Babu, R.O., Aykkal, R., Narayanan, R., 2012. Virtual screening and in vitro assay of potential drug like inhibitors from spices against glutathione-S-transferase of filarial nematodes. Journal of Molecular Modelling 18, 151–163.
Behera, D.R., Bhatnagar, S., 2019. In-vitro and in silico efficacy of isolated alkaloid compounds from Rauvolfia tetraphylla L. against bovine filarial parasite Setaria cervi: a drug discovery approach. J. Parasit. Dis. 43, 103–112.
Bennett, G., 1974. Oviposition of Boophilus microplus (Canestrini) (Acarida: ixodidae). I. Influence of tick size on egg production. Acarologia 16, 52–61.
Braz, V., Gomes, H., Galina, A., Saramago, L., Braz, G., da Silva Vaz Jr., I., Logullo, C., da Fonseca, R.N., Campos, E., Moraes, J., 2019. Inhibition of energy metabolism by 3-bromopyruvate in the hard tick Rhipicephalus microplus. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 218, 55–61.
Chen, C.-Y., Chang, F.-R., Pan, W.-B., Wu, Y.-C., 2001. Four alkaloids from Annona cherimola. Phytochemistry 56, 753–757.
Choubey, S.K., Jeyaraman, J., 2016. A mechanistic approach to explore novel HDAC1 inhibitor using pharmacophore modelling, 3D-QSAR analysis, molecular docking, density functional and molecular dynamics simulation study. J. Mol. Graph. Model. 70, 54–69.
Conceicao, R.S., Carneiro, M.M.A.d.A., Reis, I.M.A., Branco, A., Vieira, I.J.C., Braz-Filho, R., Botura, M.B., 2017. In vitro acaricide activity of Ocotea aciphylla (Nees) Mez.(Lauraceae) extracts and identification of the compounds from the active fractions. Ticks and Tick-borne Diseases 8, 275–282.
Cruz, P.B., Barbosa, A.F., Zeringóta, V., Melo, D., Novato, T., Fidelis, Q.C., Fabri, R.L., de Carvalho, M.G., Sabaa-Srur, A.U.O., Daemon, E., 2016. Acaricidal activity of methanol extract of Acmella oleracea L.(Asteraceae) and spilanthol on Rhipicephalus microplus (Acari: ixodidae) and Dermacentor nitens (Acari: ixodidae). Vet. Parasitol. 228, 137–143.
Cuevas-Hernández, R.I., Girard, R.M., Martínez-Cerón, S., Santos da Silva, M., Elias, M. C., Crispim, M., Trujillo-Ferrara, J.G., Silber, A.M., 2020. A fluorinated phenylbenzothiazole arrests the Trypanosoma cruzi cell cycle and diminishes the infection of mammalian host cells. Antimicrob. Agents Chemother. 64, 1–16.
Debnath, B., Singh, W.S., Das, M., Goswami, S., Singh, M.K., Maiti, D., Manna, K., 2018. Role of plant alkaloids on human health: a review of biological activities. Mater. Today Chem. 9, 56–72.
Divya, T., Soorya, V., Amithamol, K., Juliet, S., Ravindran, R., Nair, S., Ajithkumar, K., 2014. Acaricidal activity of alkaloid fractions of Leucas indica Spreng against Rhipicephalus (Boophilus) annulatus tick. Trop. Biomed. 31, 46–53.
Drummond, R., , et al.Ernst, S., Trevino, J., Gladney, W., Graham, O., 1973. Boophilus annulatus and B. microplus: laboratory tests of insecticides. J. Econ. Entomol. 66, 130–133.
Ferreira, G.G., do Nascimento Brandão, D.L., Dolabela, M.F., 2020. Predição do comportamento farmacocinético, toxicidade e de atividades biológicas de alcaloides isolados de Geissospermum laeve (Vell.) Miers. Research, Society Development 9, 1–23.
Ganesan, A., 2016. Multitarget drugs: an epigenetic epiphany. Chem. Med. Chem. 11, 1227–1241.
Ghosh, S., Gupta, S., Kumar, K.A., Sharma, A.K., Kumar, S., Nagar, G., Kumar, R., Paul, S., Fular, A., Chigure, G., 2017. Characterization and establishment of a reference deltamethrin and cypermethrin resistant tick line (IVRI-IV) of Rhipicephalus (Boophilus) microplus. Pestic. Biochem. Physiol. 138, 66–70.
Ghosh, S., Tiwari, S.S., Kumar, B., Srivastava, S., Sharma, A.K., Kumar, S., Bandyopadhyay, A., Julliet, S., Kumar, R., Rawat, A., 2015. Identification of potential plant extracts for anti-tick activity against acaricide resistant cattle ticks, Rhipicephalus (Boophilus) microplus (Acari: ixodidae). Exp. Appl. Acarol. 66, 159–171.
Grisi, L., Leite, R.C., Martins, J.R.d.S., Barros, A.T.M.d., Andreotti, R., Cançado, P.H.D., León, A.A.P.d., Pereira, J.B., Villela, H.S., 2014. Reassessment of the potential economic impact of cattle parasites in Brazil. Rev. Bras. Parasitol. Vet. 23, 150–156.
Guneidy, R.A., Shahein, Y.E., Abouelella, A.M., Zaki, E.R., Hamed, R., 2014. Inhibition of the recombinant cattle tick Rhipicephalus (Boophilus) annulatus glutathione S-transferase. Ticks and Tick-borne Diseases 5, 528–536.
Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M., Jakoby, W.B., 1974. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. Proceed. Nat. Academy Sci. U.S.A. 71, 3879–3882.
Hall Jr., D.C., Ji, H.-F., 2020. A search for medications to treat COVID-19 via in silico molecular docking models of the SARS-CoV-2 spike glycoprotein and 3CL protease. Trav. Med. Infect. Dis. 35, 1–13.
Hamza, I., Dailey, H.A., 2012. One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metazoans. Biochim. Biophys. Acta Mol. Cell Res. 1823, 1617–1632.
Hanwell, M.D., Curtis, D.E., Lonie, D.C., Vandermeersch, T., Zurek, E., Hutchison, G.R., 2012. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. J. Cheminf. 4, 1–17.
Hecht, D., Fogel, G.B., 2009. A novel in silico approach to drug discovery via computational intelligence. J. Chem. Informat. Modelling 49, 1105–1121.
Kaewmongkol, S., Kaewmongkol, G., Inthong, N., Lakkitjaroen, N., Sirinarumitr, T., Berry, C., Jonsson, N., Stich, R., Jittapalapong, S., 2015. Variation among Bm86 sequences in Rhipicephalus (Boophilus) microplus ticks collected from cattle across Thailand. Exp. Appl. Acarol. 66, 247–256.
Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J., 2015. The Phyre2 web portal for protein modelling, prediction and analysis. Nat. Protoc. 10, 845–858.
Klafke, G.M., Sabatini, G.A., Thais, A., Martins, J.R., Kemp, D.H., Miller, R.J., Schumaker, T.T., 2006. Larval immersion tests with ivermectin in populations of the cattle tick Rhipicephalus (Boophilus) microplus (Acari: ixodidae) from State of Sao Paulo, Brazil. Vet. Parasitol. 142, 386–390.
Kumar, R., Nagar, G., Sharma, A.K., Kumar, S., Ray, D., Chaudhuri, P., Ghosh, S., 2013. Survey of pyrethroids resistance in Indian isolates of Rhipicephalus (Boophilus) microplus: identification of C190A mutation in the domain II of the para-sodium channel gene. Acta Trop. 125, 237–245.
Kwang, L.S., 2005. In silico high-throughput screening for ADME/Tox properties: PreADMET program. Abstr Conf Comb Chem Jpn 21, 22–28.
Laskowski, R.A., Furnham, N., Thornton, J.M., 2013. The ramachandran plot and protein structure validation, biomolecular forms and functions: a celebration of 50 Years of the ramachandran map. World Scientific 1, 62–75.
Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291.
Li, H.-T., Wu, H.-M., Chen, H.-L., Liu, C.-M., Chen, C.-Y., 2013. The pharmacological activities of (−)-anonaine. Molecules 18, 8257–8263.
Lima, H.G.d., Santos, F.O., Santos, A.C.V., Silva, G.D.d., Santos, R.J.d., Carneiro, K.d.O., Reis, I.M.A., Estrela, I.d.O., Freitas, H.F.d., Bahiense, T.C., 2020. Anti-tick effect and cholinesterase inhibition caused by Prosopis juliflora alkaloids: in vitro and in silico studies. Rev. Bras. Parasitol. Vet. 29, 1–15.
Lopes, W.D.Z., Teixeira, W.F.P., de Matos, L.V.S., Felippelli, G., Cruz, B.C., Maciel, W.G., Buzzulini, C., Fávero, F.C., Soares, V.E., de Oliveira, G.P., 2013. Effects of macrocyclic lactones on the reproductive parameters of engorged Rhipicephalus (Boophilus) microplus females detached from experimentally infested cattle. Exp. Parasitol. 135, 72–78.
Mangoyi, R., Hayeshi, R., Ngadjui, B., Ngandeu, F., Bezabih, M., Abegaz, B., Razafimahefa, S., Rasoanaivo, P., Mukanganyama, S., 2010. Glutathione transferase from Plasmodium falciparum–Interaction with malagashanine and selected plant natural products. J. Enzym. Inhib. Med. Chem. 25, 854–862.
Mannervik, B., 1985. The isoenzymes of glutathione transferase. Adv. Enzymol. Relat. Area Mol. Biol. 57, 357–417.
Mannervik, B., Helena Danielson, U., Ketterer, B., 1988. Glutathione transferases—structure and catalytic activit. Crit. Rev. Biochem. 23, 283–337.
Moraes, J., Arreola, R., Cabrera, N., Saramago, L., Freitas, D., Masuda, A., da Silva Vaz Jr., I., de Gomez-Puyou, M.T., Perez-Montfort, R., Gomez-Puyou, A., 2011. Structural and biochemical characterization of a recombinant triosephosphate isomerase from Rhipicephalus (Boophilus) microplus. Insect Biochem. Mol. Biol. 41, 400–409.
Ndawula Jr., C., Sabadin, G.A., Parizi, L.F., Vaz Jr., I.S., 2019. Constituting a glutathione S-transferase-cocktail vaccine against tick infestation. Vaccine 37, 1918–1927.
Olivares-Illana, V., Pérez-Montfort, R., López-Calahorra, F., Costas, M., Rodríguez-Romero, A., Tuena de Gómez-Puyou, M., Gómez Puyou, A., 2006. Structural differences in triosephosphate isomerase from different species and discovery of a multitrypanosomatid inhibitor. Biochemistry 45, 2556–2560.
Ozelame, K.P.C., Mattia, M.M.C., Silva, L.A.D., Randall, L.M., Corvo, I., Saporiti, T., Seixas, A., da Silva Vaz Jr., I., Alvarez, G., 2022. Novel tick glutathione transferase inhibitors as promising acaricidal compounds. Ticks and Tick-borne Diseases 13, 1–7.
Pereira, G., Bruno, 2021. In Silico Pharmacology Studies of Phenolic Esters Designed to Obtain Tyrosine Kinase Inhibitors. Chemistry department. Federal Technological University of Paraná, Campo Mourão- PR., pp. 1–67
Prade, L., Huber, R., Manoharan, T.H., Fahl, W.E., Reuter, W., 1997. Structures of class pi glutathione S-transferase from human placenta in complex with substrate, transition-state analogue and inhibitor. Structure 5, 1287–1295.
Roche, J., Bertrand, P., 2016. Inside HDACs with more selective HDAC inhibitors. Eur. J. Med. Chem. 121, 451–483.
Roditakis, E., Roditakis, N.E., Tsagkarakou, A., 2005. Insecticide resistance in Bemisia tabaci (Homoptera: aleyrodidae) populations from crete. Pest Manag. Sci. 61, 577–582.
Rosado-Aguilar, J., Arjona-Cambranes, K., Torres-Acosta, J., Rodríguez-Vivas, R., Bolio-González, M., Ortega-Pacheco, A., Alzina-López, A., Gutiérrez-Ruiz, E., Gutiérrez-Blanco, E., Aguilar-Caballero, A., 2017. Plant products and secondary metabolites with acaricide activity against ticks. Vet. Parasitol. 238, 66–76.
Saramago, L., Gomes, H., Aguilera, E., Cerecetto, H., González, M., Cabrera, M., Alzugaray, M.F., Vaz Junior, I.S., Nunes da Fonseca, R., Aguirre-López, B., 2018. Novel and selective Rhipicephalus microplus triosephosphate isomerase inhibitors with acaricidal activity. Veterinary Sci. 5, 1–19.
Shen, W. J. Zhang, Fang, H., Perkins, R., Tong, W., Hong, H., 2013. Homology modelling, molecular docking, and molecular dynamics simulations elucidated α-fetoprotein binding modes. BMC Bioinf. 14, 1–14.
Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 1–6.
Silva, G.D., de Lima, H.G., de Freitas, H.F., da Rocha Pita, S.S., dos Santos Luz, Y., de Figueiredo, M.P., Uzeda, R.S., Branco, A., Costa, S.L., Batatinha, M.J.M., 2021. In vitro and in silico studies of the larvicidal and anticholinesterase activities of berberine and piperine alkaloids on Rhipicephalus microplus. Ticks and Tick-borne Diseases 12, 1–6.
Tavares, C.P., Sousa, I.C., Gomes, M.N., Miró, V., Virkel, G., Lifschitz, A., Costa-Junior, L. M., 2022. Combination of cypermethrin and thymol for control of Rhipicephalus microplus: efficacy evaluation and description of an action mechanism. Ticks and Tick-borne Diseases 13, 1–8.
Tong, J.-B., Luo, D., Bian, S., Zhang, X., 2021. Structural investigation of tetrahydropteridin analogues as selective PLK1 inhibitors for treating cancer through combined QSAR techniques, molecular docking, and molecular dynamics simulations. J. Mol. Liq. 335, 1–17.

Van De Waterbeemd, H., Gifford, E., 2003. ADMET in silico modelling: towards prediction paradise? Nat. Rev. Drug Discov. 2, 192–204.
Vaz Jr., I.S., Lermen, T.T., Michelon, A., Ferreira, C.A.S., de Freitas, D.R.J., Termignoni, C., Masuda, A., 2004. Effect of acaricides on the activity of a Boophilus microplus glutathione S-transferase. Vet. Parasitol. 119, 237–245.
Wadapurkar, R.M., Shilpa, M., Katti, A.K.S., Sulochana, M., 2018. In silico drug design for Staphylococcus aureus and development of host-pathogen interaction network. Inform. Med. Unlocked 10, 58–70.
Wadood, A., Ahmed, N., Shah, L., Ahmad, A., Hassan, H., Shams, S., 2013. In-silico drug design: an approach which revolutionarised the drug discovery process. OA Drug Design and Delivery 1, 1–4.
Xavier, M., Sehnem Heck, G., Boff de Avila, M., Maria Bernhardt Levin, N., Oliveira Pintro, V., Lemes Carvalho, N., Filgueira de Azevedo, W., 2016. SAnDReS a computational tool for statistical analysis of docking results and development of scoring functions. Comb. Chem. High Throughput Screening 19, 801–812.
Yee, S., 1997. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharmaceut. Res. 14, 763–766.
Yusuf, D., Davis, A.M., Kleywegt, G.J., Schmitt, S., 2008. An alternative method for the evaluation of docking performance: RSR vs RMSD. Journal of Chemical Information and Modelling 48, 1411–1422.