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

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-

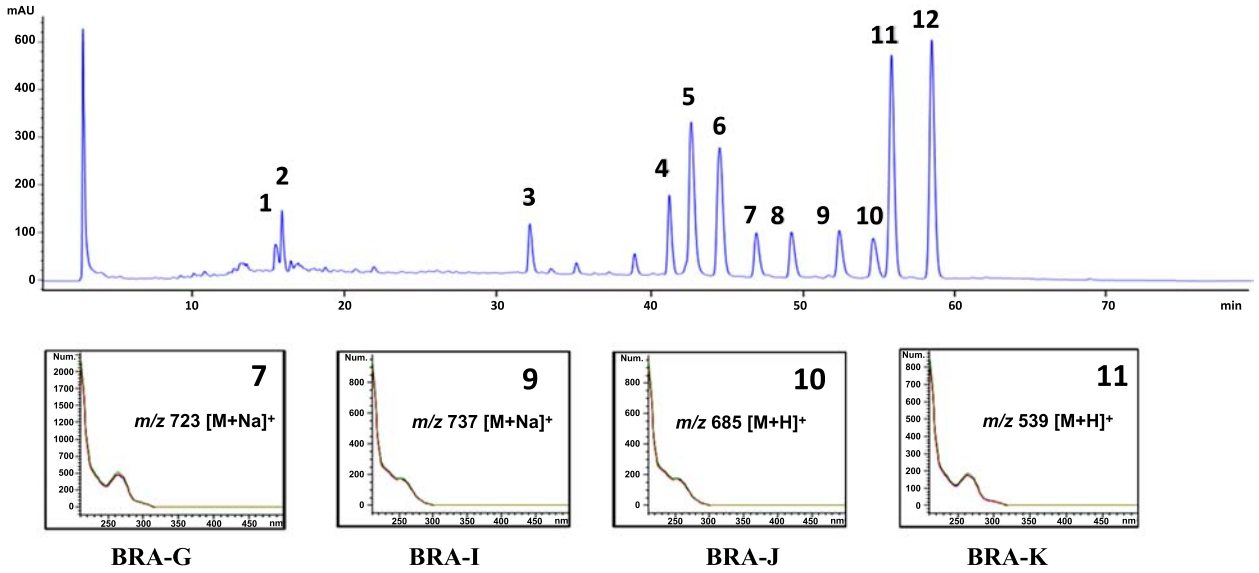

Fig. (1). HPLC-UV/MS analysis of the hydroethanolic extract from *Fridericia platyphylla* at 254 nm and identification of brachydins G, I, J, and K, adapted from Da Rocha *et al*. [22]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

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.

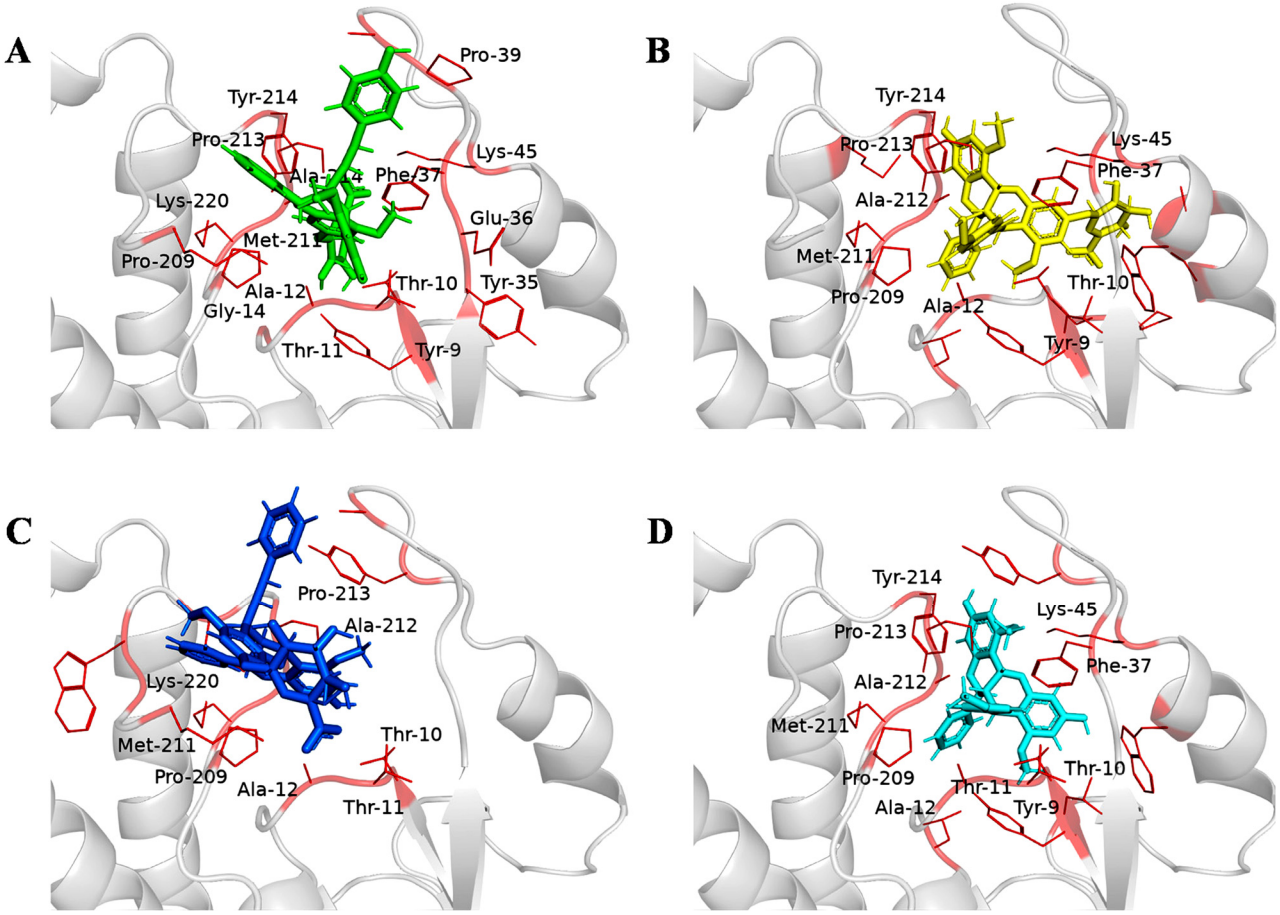

Fig. (2). Cartoon representation of GST of *Rhipicephalus microplus* in complex with **A**) BRA-G; **B**) BRA-I; **C**) BRA-J; and **D**) BRA-K. The ligands are shown as sticks. In red, GST residues within 3.5 Å of brachydins. (*A higher resolution / colour version of this figure is available in the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

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