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Antileishmanial activity *in silico* and *in vitro* of semi-synthetic derivatives obtained from natural products

Atividade antileishmania *in silico* e *in vitro* de derivados semissintéticos obtidos de produtos naturais

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American cutaneous leishmaniasis (ACL) continues to be a public health problem in at least 85 countries around the world. Since medications for the treatment of ACL have toxic side effects, there is a need to search for new active molecules from plants. The objective of this study was to investigate the *in vitro* and *in silico* antileishmanial activity of semi-synthetic derivatives obtained from natural products. The bioassay used the species of *Leishmania* (*L.*) *amazonensis* and *Leishmania* (*V.*) *guyanensis* cultivated in supplemented RPMI medium. The 50% inhibitory concentration (IC_{50}) was obtained by inhibiting promastigotes exposed to the solutions naringenin, aminoguanidine, CNFD, FGS-3, FGS-16 and FGS-18 at concentrations of 50 to 3.125 $\mu g.mL^{-1}$. In the experiment, 0.2% DMSO and pentamidine isethionate at 3 $\mu g.mL^{-1}$ were used as the controls for 24, 48 and 72 h, in triplicate. Regarding the interaction of the active ingredients with the enzymatic targets (2WP5 and 2VPM), molecular docking was used with the aid of the AutoDock Vina v.1.2.5. As for the derivatives FGS-16 and FGS-18, these presented spectra within those described in the literature. Regarding the 50% cytotoxic concentration (CC_{50}) in cells after 72 h, FGS-18=329.9±0.3 $\mu g.mL^{-1}$. The IC_{50} of FGS-16=0.7±0.2 $\mu g.mL^{-1}$ for *L. guyanensis* and 0.5±0.6 $\mu g.mL^{-1}$ for *L. amazonensis*.Therefore, the selectivity index was 45.7 (*L. guyanensis*) and 64 (*L. amazonensis*). Furthermore, the molecule presented three hydrogen bonds for the 2WPM target. Therefore, the FGS-16 proved to be the most promising derivative with antileishmanial activity.
Keywords: *Leishmania*, molecular docking, natural products.

A leishmaniose tegumentar americana (LTA) ainda é um problema de saúde pública em pelo menos 85 países ao redor do mundo. Visto que os medicamentos para o tratamento da LTA apresentam efeitos colaterais tóxicos, havendo a necessidade de buscar novas moléculas ativas a partir de plantas. O objetivo deste estudo foi investigar a atividade antileishmania *in vitro* e *in sílico* de derivados semissintéticos obtidos de produtos naturais. No bioensaio, foram usadas as espécies de *Leishmania* (*L.*) *amazonensis* e *Leishmania* (*V.*) *guyanensis* cultivados em meio RPMI suplementado. A concentração inibitória 50% (IC_{50}) foi obtida pela inibição dos promastigotas expostos as soluções de amirona, naringenina, aminoguanidina, naftaquinona, FGS-16 e FGS-18 nas concentrações de 50 a 3.125 $\mu g.mL^{-1}$. No experimento, usou-se como controles o DMSO 0.2% e o isetionato de pentamidina a 3 $\mu g.mL^{-1}$ durante 24, 48 e 72 h, em triplicata. Quanto à interação dos ativos com os alvos enzimáticos (2WP5 e 2VPM), usou-se docking molecular com o auxílio o AutoDock Vina v.1.2.5. Quanto aos derivados, FGS-16 e FGS-18 apresentaram espectros dentro do descrito na literatura. Acerca da concentração citotóxica 50% (CC_{50}) em células por 72 h, a FGS-18=329.9±0.3 $\mu g.mL^{-1}$. A CI_{50} da FGS-16=0.7±0.2 $\mu g.mL^{-1}$ para *L. guyanensis* e de 0.5±0.6 $\mu g.mL^{-1}$ para *L. amazonensis*. Além disso, o índice de seletividade foi de 45.7 (*L. guyanensis*) e de 64 (*L. amazonensis*). Ademais, a molécula apresentou três ligações de hidrogênio ao alvo 2WPM. Portanto, a FGS-16 se mostrou mais promissora com atividade antileishmania.
Palavras-chave: *Leishmania*, docagem molecular, produtos naturais.

1. INTRODUCTION

Leishmaniasis is a vector-borne disease that is part of the group of neglected infectious diseases. It occurs in the poorest countries and affects the most vulnerable populations that have difficult access to health services. These diseases have a wide spectrum of clinical manifestations, and the differences in these manifestations are related to the species of *Leishmania* involved [1]. American cutaneous leishmaniasis (ACL) is a public health problem in more than 85 countries, with 0.7 to 1.3 million new cases reported annually. This disease has a high infection detection rate it is considered by the World Health Organization as one of the six most important infectious diseases [1]. In Brazil, in 2019, the incidence of ACL presented an annual record of 15,922 thousand cases, with 1,315 of these in the state of Amazonas and 506 in Manaus alone [2].

Currently, the drugs used in the therapy for the various forms of leishmaniasis are pentavalent antimonials. Despite being toxic, they are still used as first choice drugs; however, new pharmaceutical formulations are being explored, for example amphotericin B, pentamidine, miltefosine and paromomycin, though it has not been possible to develop a new, more effective and less toxic drug [3]. In addition to the therapeutic restrictions for pregnant women and the elderly, severe toxicity (cardiotoxic, hepatotoxic and nephrotoxic), high cost, low efficacy, among others [4]. New strategies to combat and control this important neglected disease are urgently needed, and one of the alternatives is the use of active ingredients obtained from plants, which have chemical compounds that have an enormous structural and functional chemical variability due to the metabolic flexibility that is necessary for these plants to adapt to various situations of environmental stress. These processes are important for the discovery of new potentially pharmacological molecules [5]. Another way to obtain new assets would be by synthesis or semi-synthesis, among others [6].

Given the biodiversity found in the five major Brazilian biomes, such as the Amazon Rainforest, the Cerrado, the Atlantic Forest, the Pantanal and the Caatinga, the plants found within them are possible sources of bioactives with potential biological and pharmacological activities, and can play various roles for the benefit of human health [7]. Many scientific studies have demonstrated the promising biological activities of these actives for various pathologies, including leishmaniasis [8]; such as the study of the *in vitro* and *in vivo* antileishmanial activity of *Libidibia ferrea* [9] among other studies.

In this context, the modification of these natural actives into new molecules, as in the study of the antileishmanial and cytotoxic activity of dillapiole *n*-butyl ether [10], can be an important and promising tool to generate prototypes of active drugs. Furthermore, to better understand the action of these molecules in a specefic inhibitory site of the parasite, there is the study of molecular modeling (docking), which is a computational technique that simulates a ligand and target interaction, thus facilitating the process of screening these molecules and their possible mechanism of action [11].

Given the above, the objective of the present study was to compare, via *in vitro* and *in silico* analyses, the antileishmanial activity of natural and semi-synthetic substances that have already demonstrated different biological activities in previous studies by our research group.

2. MATERIALS AND METHODS

2.1 Acquisition of commercial samples

Aminoguanidine and naringeninine were purchased from Sigma-Aldrich, with purity levels of 97% and > 95%, respectively.

2.2 Synthesis of FGS-3, FGS-16 and FGS-18

These samples were synthesized from the isomeric mixture of α, β-amyrin (FGS-1) isolated from the bark of the stem of *Byrsonima* spp*.,* as described in the work of Guilhon-Simplicio et al. (2022) [12]. For this, stem bark of the species *B. crispa*, *B. duckeana*, *B. garcibarrigae* and

B. incarnata was collected in the Adolpho Ducke Forest Reserve, Manaus, Amazonas. The collection of samples was authorized by the Chico Mendes Institute for Biodiversity Conservation under registration number 41553-1. The species was identified by botanists of the herbarium at the National Institute for Amazonian Research (INPA), within the scope of the project "Flora of the Ducke Reserve" and the respective exsiccates were deposited under numbers 1177, 1163, 691, 778. After collection, the samples were placed for drying in an air-circulation oven, at a temperature of 40-60 °C. The dried bark was then ground in a knife mill and the resulting powder was kept at room temperature and away from light. For the characterization of the raw material, the powder was subjected to granulometric analysis in a sieve, according to the method of the Brazilian Pharmacopoeia.

The mixture of α, β-amyrin (FGS-1), its derivatives and the NMR spectra were obtained following the methodology of Guilhon-Simplicio et al. (2022) [12]. The reagents and solvents used to obtain FGS-3, FGS-18 and FGS-16 were predominantly of analytical grade. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were obtained in a spectrometer (Bruker, Billerica) at frequencies of 400 MHz using $CDCl_3$ or deuterated DMSO as the solvent. NMR spectra were processed with the 1D NMR Processor version 12.0 program and chemical shifts were compared with literature data [12]. TMS was used as a reference for the chemical shifts. Thin-layer chromatography (TLC) was carried out using silica gel 60 F254 0.20 mm precoated aluminum sheets (Alugram, Macherey-Nagel).

Isolation of α, β-amyrin (FGS-1) was achieved from stem bark extracts of *Byrsonima spp*., obtained via kinetic maceration with hexane, followed by filtration and then concentrated under reduced pressure. The hexane extracts were washed with acetone, centrifuged, and the resulting precipitate was collected. This procedure was then repeated using methanol. The product was identified as α, β-amyrin (FGS-1).

The derivative 3-oxo α, β-amyrin was obtained by dissolving FGS-1 (100 mg; 0.23 mmol) in 10 mL of dichloromethane, then adding pyridinium chlorochotmate (70 mg, 0.32 mmol); thereafter the mixture was stirred at room temperature for 24 h. The product was washed with diethyl ether on gauze and cotton, dried over anhydrous sodium sulfate, and the solvent was evaporated in an exhaust hood. The product was purified on a silica gel chromatographic column with hexane: ethyl acetate (9:1–8:2) as the eluent, yielding 70 mg (70%). The product was identified as FGS-3.

To obtain FGS-16 (3-guanidine α, β-amyrin), FGS-3 (66.8 mg; 0.15 mmol) was dissolved in 10 mL of ethanol, 1 mL of water and 1 mL of hydrochloric acid, and 50.8 mg (0.45 mmol) of aminoguanidine hydrochloride was added, then the compound was stirred for 6 h at room temperature. The solvent was evaporated, and the residue was dissolved in 20 mL of water and extracted 3 times with 20 mL of ethyl acetate. The organic phase was washed twice with 20 mL of saturated sodium bicarbonate solution, and the aqueous phase was extracted three times with 20 mL of ethyl acetate. The organic phase was washed with 20 ml of water, dried over anhydrous sodium sulfate and evaporated. The residue was dissolved in 10 ml of ethanol, then 1 ml of 1 N hydrochloric acid was added, and the compounds were stirred for 1 h [13]. The solvent was evaporated in an exhaust hood, and the residue was recrystallized from ethanol, in a yield of 86%.

The oxime derivative (FGS-18) was obtained by solubilizing FGS-3 (51.6 mg; 0.12 mmol) and hydroxylamine hydrochloride (25.3 mg; 0.76 mmol) in 2 mL of pyridine and stirring at 60 °C for 4 h. Subsequently, the compound was extracted with dichloromethane (10 mL) and washed three times with 5 mL of 10% hydrochloric acid. The organic phase was dried over anhydrous sodium sulfate and evaporated in an exhaust hood [14]. The product was purified in a silica gel chromatographic column with hexane/ethyl acetate (9:1-8:2) as the eluent, yielding 100%.

2.3 Síntese of the CNFD derivative

The synthesis of the CNFD was performed as described by Freire and Azevedo (2010) [6]. According to the authors, the synthesis of naphthoquinone was prepared from the readily available lawsone and olefins in the presence of cerium (IV) and ammonium nitrate (CAN) and was carried out in a single step. The compounds (furan naphthoquinone) were obtained by oxidation and cyclo-addition of 2-hydroxy-1,4-naphthoquinone (lawsone) to the alkene, mediated by cerium (IV) and CAN. All the compounds were obtained with good yields (10-86% of product isolates)

and were completely characterized using proton and carbon spectroscopy, nuclear magnetic resonance, infrared spectroscopy and elemental analysis.

2.4 Acquisition of the J774 macrophages

The J774 macrophages were removed from the cryopreservation at -80 ºC. The cells were cultured in a 96-well plate in RPMI-1640 medium containing 20% fetal bovine serum (FBS), 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen). For the cytotoxicity test, a cell concentration of $5x10^6$ (adjusted in a Neubauer chamber) was used. The cells were plated in triplicate and, after 24 h of incubation, they were exposed to the actives (FGS-16, FGS-18, aminoguanidine, naringenin, amyrone and naphthaquinone) in serial concentrations of 50 to 1.5 µg mL^{-1}. The controls were pentamidine isethionate and DMSO (0.2%) plus cells. After 72 h, the bioassay underwent a dose-response analysis.

2.5 Acquisition and maintenance of the *Leishmania* strains

The following *Leishmania* strains were used: *Leishmania* (*V.*) *guyanensis* (MHO/BR/75/M4147) and *Leishmania* (*L.*) *amazonensis* (MHOM/BR/2009/IM 5584), which were cryopreserved at the Laboratory of Leishmaniasis and Chagas Disease, INPA. Subsequently, these were cultured in RPMI 1640 medium supplemented with inactivated fetal bovine serum (iBFS) and incubated in an oven at 25 ºC.

2.6 Determination of antileishmanial activity against promastigote forms

The actives FGS-3, FGS-16, FGS-18, aminoguanidine (AGD), naringenin (NRG), and 6b,7-dihydro-5H-cyclopenta[b]naphtho[2,1-d]furan-5,6(9aH)-dione (CNFD) were evaluated according to growth inhibition and mortality of promastigote forms of *L. (V.) guyanensis* and *L. (L.) amazonensis*. Before each experiment, flagellar motility and general morphology of parasites were observed. The metacyclic promastigote forms were centrifuged at 4,400 rpm for 15 min, washed in sterile saline, diluted and counted in a Neubauer chamber and adjusted to obtain the parasite concentration of 10^6 parasites mL^{-1}. The actives were weighed and diluted in DMSO 0.2% (negative control). Subsequently, the parasites were plated in a 96-well plate, then the molecules were added from 50 µg to 3.125 µg.mL^{-1}. As a positive control, pentamidine isethionate at 3 µg.mL^{-1} was used for 24, 48 and 72 h, in triplicate. The bioassay was placed in an oven at 25 ºC. After these periods, parasitic viability was evaluated by counting live promastigotes (evaluating cell motility) in a Neubauer chamber under an optical microscope with a magnification of 400x. The average number of live promastigotes was used to calculate the linear regression of the IC_{50}, and these data were used to express the results of the activity of the evaluated samples and, thus, select the bioactive(s) and the most promising concentration.

2.7 Molecular docking

To investigate a possible interaction between the bioactives and the enzyme targets of interest in this study, the preparation of the structures of the receptors and ligands was carried out, with the molecular docking protocols being based on Forli et al. (2016) [15]. The 3D structure of the enzyme triphathion reductase and the triphathion synthetase were obtained from the Protein Data Bank (PDB) [16] with the PDB IDs 2WP5 [17] and 2VPM [18] respectively. The coordinates of the binding site for 2WP5 were center_x = -23.333437; center_y = -29.646307; center_z = 60.193077; while, for 2VPM, these were as follows: center_x = -21.163086; center_y = -1.6194364; center_z = 33.79551. Then, with the help of the Schrodinger Maestro 2021-2 software [19], water molecules, co-crystallized ligands and any heteroatoms not involved in the bond were removed; in addition, hydrogen atoms not captured by crystallography were added, as well as all the figures of the complex that had been constructed through it. The chemical structures of the molecules FGS-3, FGS-16, FGS-18, naringenin, pentamidine isethionate, aminoguanidine

and CNFD were designed in MarvinSketch v. 23.17 from chemical databases and others manually, with the three-dimensional geometry of the ligand being optimized via the MMFF94 method. After this, an automated script in Python was used, through the script prepare_receptor4.py and prepare_ligand4.py, by which ligands and receptors were automatically converted to the format .pdbqt required for molecular docking in the AutoDock algorithm Vina v. 1.2. 5 [20], using the completeness parameter equal to 96. The diagrams of chemical interactions in the protein-ligand complex were constructed using the BIOVIA Discovery Studio 2021 software. Re-docking was also performed using the same molecular docking protocols used in this research for validation. The structure chosen for analysis was triphathione reductase (PDB ID: 2WP5), which already has a co-crystallized ligand, whose inhibitor is a 3,4-dihydroquinazoline. The structural alignment was done using the Python tool known as OpenBabel.

2.8 Statistical analysis

The IC_{50} values were obtained by linear regression, inhibition analysis and statistical differences between concentrations and controls, analyzed using ANOVA followed by the Tukey test with 95% confidence. The analyses were performed in GraphPad Prism 6.0.

3. RESULTS

3.1 Derivatives of FGS-1

The FGS-3, FGS-16 and FGS-18 was successfully obtained from FGS-1, in according to described previously. The shifts observed in the NMR agreed with the data found in the literature [12]. Chemical modifications were carried out at the C-3 position of the triterpenic structure, as shown in Figure 1.

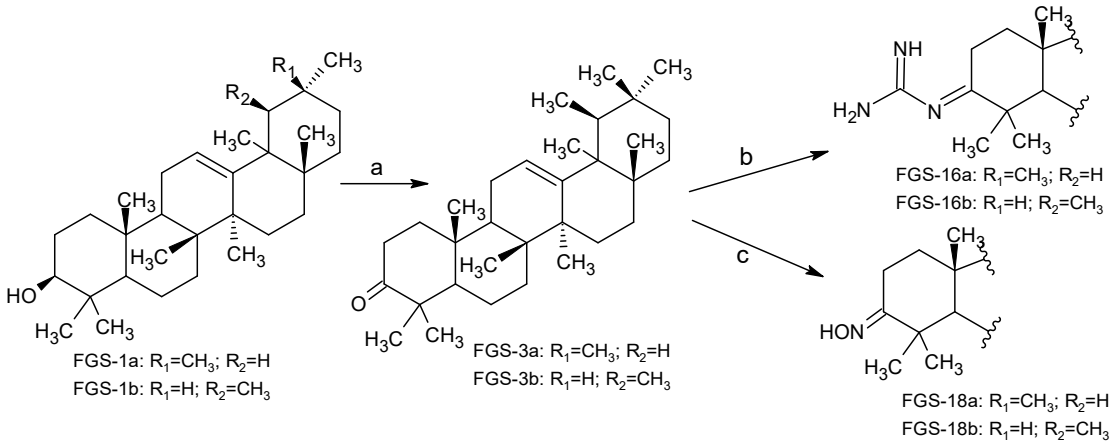

*Figure 1: Scheme of the semi-synthesis of the oxidized derivative of α, β-amyrin (**FGS-3**) and from this, nitrogen derivatives (**FGS-16** and **FGS-18**). (a) Jones reagent, acetone, rt 24 h; (b) aminoguanidine hydrochloride, HCl, EtOH, H_2O, reflux 6 h.; (c) $HONH_2$.HCl, pyridine, reflux 4 h.*

3.2 Cellular cytotoxicity

In the cytotoxicity assay with the J774 macrophages exposed to the actives FGS-3, FGS-16, FGS-18, aminoguanidine (AGD), naringenin (NRG), and 6b,7-dihydro-5H-cyclopenta[b] naphtho[2,1-d]furan-5,6 (9aH)-dione (CNFD) at concentrations of 1.5 to 50 µg.mL^{-1} for 72 h, it was observed that CNFD was very toxic in relation to the other molecules, with a CC_{50} of 2.4 ± 0.8 µg.mL^{-1} when compared with the negative control (DMSO at 0.2%). This result was also seen in the study of De Almeida et al. (2021) [21], who observed a CC_{50} of 3.76 µm in human fibroblast

cells (MRC5). As for the other molecules, these showed the following CC_{50} in $\mu g.mL^{-1}$; FGS-3 = 168.9 ± 0.4, FGS-16 = 32 ± 0.4; FGS-18 = 329 ± 0.3; AGD = 70 ± 0.1; NRG = 169.7 ± 0.5. It is noteworthy that the molecules FGS-16 and FGS-18 are unpublished antileishmanial derivatives. In the preparation for the synthesis of FGS-16, the molecules amyrenone plus aminoguanidine were used in the reaction, which showed that the derivative presented a more toxic profile than its precursors, whereas FGS-18 has in its synthesis the molecules amyrone plus hydroxylamine. Even though amyrone presented a non-toxic CC_{50} at the concentration used, the derivative showed a higher value of cellular toxicological safety. This suggests that the synthesis of FGS-18 showed a safe CC_{50}, as shown in Figure 2.

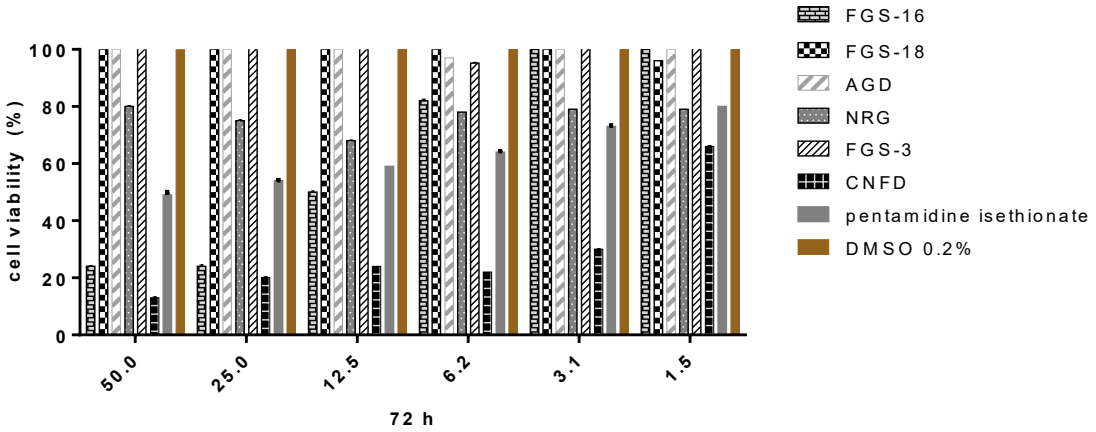

Figure 2. Analysis of the viability of J774 macrophages when exposed to the bioactives FGS-16, FGS-18, aminoguanidine (AGD), naringenin (NRG), amyrone (FGS-3) and 6b,7-dihydro-5H-cyclopenta[b] naphtho[2,1-d]furan-5,6(9aH)-dione (CNFD) at concentrations of 50 to 1.5 $\mu g.mL^{-1}$ for 72 h, pentamidine isethionate (positive control) and 0.2% DMSO (negative control). Results are the averages of three repetitions.

3.3 Antileishmanial activity of the substances

In the results obtained from the bioactives with antileishmanial activity, aminoguanidine (AGD) and amyrone (FGS-3) at serial concentrations of 50 to 3.1 $\mu g.mL^{-1}$ against the promastigote forms of *L.* (*V.*) *guyanensis*, as shown in Figure 3, AGD showed an IC_{50} of 85.6 ± 1.5 $\mu g.mL^{-1}$ at 24 h and of 62.2 ± 2.5 $\mu g.mL^{-1}$ at 72 h. However, the concentration of 50 $\mu g.mL^{-1}$ was significant in all other lower concentrations when compared with the negative control (DMSO 0.2%) (Figure 3A). FGS-3 showed an IC_{50} of 104.8 ± 1.7 and 236.1 ± 1.5 $\mu g.mL^{-1}$ at 24 and 72 h respectively, in addition to demonstrating significance at the concentration of 50 $\mu g.mL^{-1}$ compared to DMSO 0.2% at 72 h (Figure 3C). For the species of *L.* (*L.*) *amazonensis*, AGD showed an IC_{50} of 187.7 ± 1.3 and 82.2 ± 2.0 $\mu g.mL^{-1}$ at the intervals of 24 and 72 h respectively, and had a $P < 0.0001$ at the concentration of 50 $\mu g.mL^{-1}$ when compared with the negative control (DMSO 0.2%) and the other lower concentrations (Figure 3B). In relation to FGS-3, this obtained an IC_{50} of 85.8 ± 3.0 at 24 h and of 39.1 ± 2.5 $\mu g.mL^{-1}$ at 72 h, in addition to demonstrating significance at the three highest concentrations when compared to DMSO 0.2% at 72 h (Figure 3D).

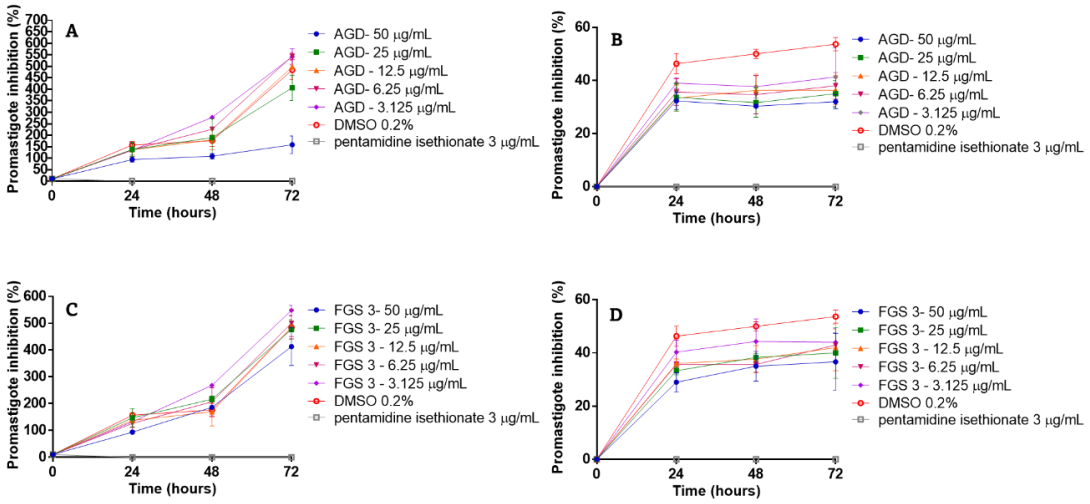

Figure 3. Antileishmanial activity of the bioactives aminoguanidine (AGD) and amyrone (FGS-3) at concentrations from 50 to 3.125 µg.mL^{-1} against the promastigote forms of Leishmania (V.) guyanensis (A and C) and Leishmania (L.) amazonensis (B and D) at 24, 48 and 72 h, pentamidine isethionate (positive control) and DMSO at 0.2% (negative control). The results were obtained via ANOVA (two-way) and Bonferroni's correction with 95% confidence using the GraphPad Prism 6® program. The tests were performed in triplicate.

Figure 4 shows the results obtained using the derivatives FGS-16 and FGS-18 against *Leishmania*. It was observed that FGS-16 presented an IC_{50} of 9.7 ± 0.3 µg.mL^{-1} at 24 h and of 0.7 ± 0.2 µg.mL^{-1} at 72 h, showing significance in all concentrations with a $P < 0.0001$, compared to DMSO 0.2%, and at all the intervals (Figure 4A). The FGS-18 molecule showed an IC_{50} of 46.9 ± 0.2 and 47.3 ± 0.2 µg.mL^{-1} at 24 and 72 h, respectively, and the concentration of 50 µg.mL^{-1} obtained a $P < 0.0001$ at the lower concentrations compared to DMSO 0.2% in the inhibition of promastigote forms of *L. guyanensis* (Figure 4C). Regarding its antileishmanial activity against *L. amazonensis*, FGS-16 showed an IC_{50} of 1.0 ± 0.5 µg.mL^{-1} at 24 h, and of 0.5 ± 0.6 µg.mL^{-1} at 72 h, and had a $P < 0.0001$ at the highest concentration, compared to DMSO 0.2%, (Figure 4B). The derivative FGS-18 presented an IC_{50} of 13.3 ± 0.1 µg.mL^{-1} at 24 h and 39.6 ± 0.3 µg.mL^{-1} at 72 h, showing significance at all concentrations, especially in the concentration of 50 µg.mL^{-1} compared to DMSO 0.2% at 72 h (Figure 4D).

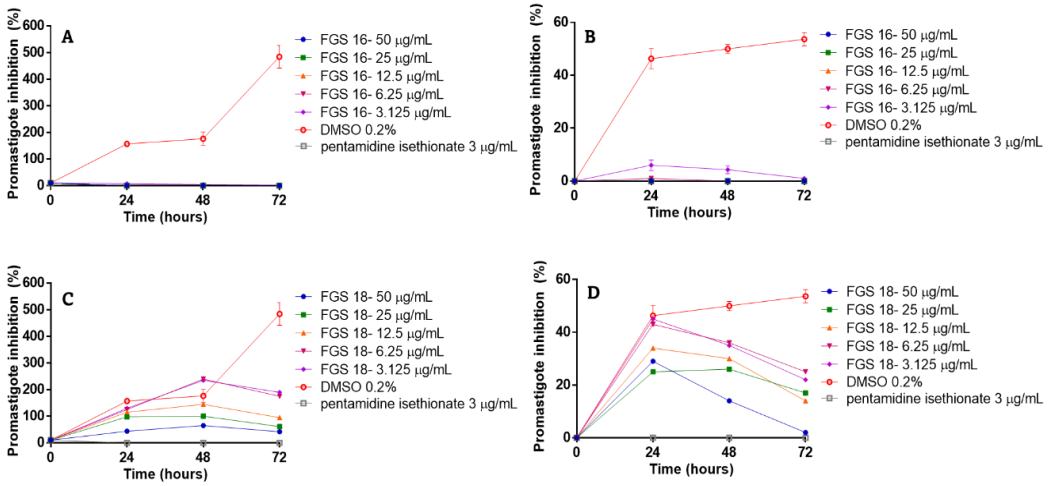

Figure 4. Antileishmanial activity of the derivatives FGS-16 and FGS-18 at concentrations from 50 to 3.125 µg.mL^{-1} against promastigote forms of L. (V.) guyanensis (A and C) and L. (L.) amazonensis (B and D) at 24, 48 and 72 h, pentamidine isethionate (positive control) and 0.2% DMSO (negative control). The results were obtained via ANOVA (two-way) and Bonferroni's correction with 95% confidence using the GraphPad Prism 6® program. The tests were performed in triplicate.

The antileishmanial activity of 6b,7-dihydro-5H-cyclopenta[b]naphtho[2,1-d]furan-5,6(9aH)-dione (CNFD) and naringenin (NRG) at serial concentrations of 50 to 3.1 $\mu g.mL^{-1}$ against promastigote forms of *Leishmania* can be seen in Figure 5. The CNFD derivative showed an IC_{50} of 25.5 ± 4.3 $\mu g.mL^{-1}$ at 24 h and of 17 ± 5.4 $\mu g.mL^{-1}$ at 72 h, and only the latter concentration did not show a $P < 0.0001$ when compared to DMSO 0.2% (Figure 5A). The NRG molecule, on the other hand, presented an IC_{50} of 84 ± 4.1 $\mu g.mL^{-1}$ and 65.9 ± 5.2 $\mu g.mL^{-1}$ at 24 and 72 h, respectively, and obtained a $P < 0.0001$ at the highest concentration over the other concentrations and DMSO 0.2% (Figure 5C) in the inhibition of *L. guyanensis*. In the inhibition of *L. amazonensis*, CNFD showed an IC_{50} of 1.0 ± 2.6 and 1.2 ± 2.3 $\mu g.mL^{-1}$ at 24 and 72 h, respectively, and was significant at all concentrations and intervals compared to DMSO 0.2% (Figure 5B). The NRG molecule presented an IC_{50} of 92.1 ± 2.1 at 24 h and 37.5 ± 4.1 at 72 h, obtained at the concentration of 50 $\mu g.mL^{-1}$ and was significant over all the other concentrations and DMSO 0.2% (Figure 5D).

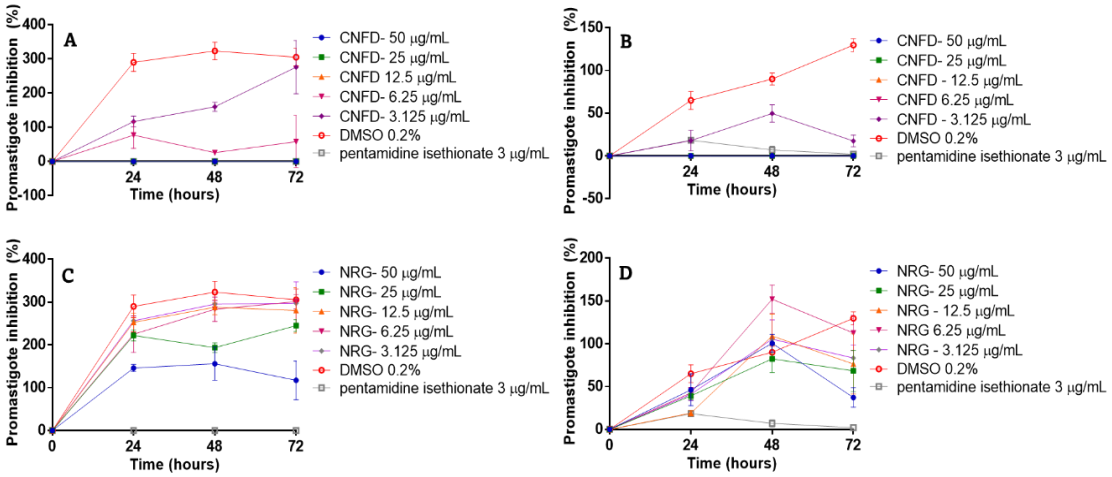

Figure 5. Antileishmanial activity of 6b,7-dihydro-5H-cyclopenta[b]naphtho[2,1-d]furan-5,6 (9aH)-dione (CNFD) and naringenin (NRG) at concentrations from 50 to 3.125 $\mu g.mL^{-1}$ against promastigote forms of L. (V.) guyanensis (A and C) and L. (L.) amazonensis (B and D) at 24, 48 and 72 h, pentamidine isethionate (positive control) and 0.2% DMSO (negative control).

3.4 Selectivity index

The results were obtained from the cytotoxic concentration 50%/parasitic concentration 50% (CC_{50}/IC_{50}) of the molecules that showed antileishmanial activity and their respective toxic profiles in macrophages, the derivatives, FGS-16 against *L. guyanensis* and *L. amazonensis*, showed better results with a non-toxic profile for cells compared to the other molecules tested, with an IS = 45.7 and 64 per species respectively. Although the FGS-18 derivative did not show high antileishmanial activity, it exhibited a non-toxic profile for cells, as it showed an IS = 8.3, when compared with the IS = 0.3 of pentamidine isethionate for *L. amazonensis* (Table 1).

Table 1. IC_{50} values for promastigotes of Leishmania (V.) guyanensis and Leishmania (L.) amazonensis and CC_{50} for J774 macrophages, and their respective selectivity index (SI) at 72 h. The means of the IC_{50} and CC_{50} are represented in $\mu g.mL^{-1}$.

	CC_{50}	IC_{50}		SI	
Samples	**J774**	[1] *L. guyanensis*	[2] *L. amazonensis*	**1**	**2**
FGS-3	168.9 ± 0.4	236.1 ± 1.5	39.1 ± 2.5	0.7	4.3
Aminoguanidine	70 ± 0.1	62.2 ± 2.5	82.2 ± 2.0	1.1	0.8
FGS-16	32 ± 0.4	0.7 ± 0.2	0.5 ± 0.6	45.7	64
FGS-18	329 ± 0.3	47.3 ± 0.2	39.6 ± 0.3	6.9	8.3
Naphthaquinone (CNFD)	2.4 ± 0.8	17 ± 5.4	1.2 ± 2.3	0.1	2
Naringenin	169.7 ± 0.5	65.9 ± 5.2	37.5 ± 4.1	2.5	4.5
pentamidine isethionate	1.2 ± 1.0	0.1 ± 1.2	4 ± 1.0	12	0.3

IC_{50} = 50% inhibitory concentration
CC_{50} = 50% cytotoxic concentration
SI= selectivity index

3.5 Molecular docking

Molecular docking analysis suggests that the active ingredients that showed the best interactions with the active site of *Leishmania infantum* 2WP5 were the molecules FGS-16, FGS-3, FGS-18 and CNFD respectively, when compared with the results of the reference drug, pentamidine isethionate. As for the 2VPM target, the best interaction was shown by FGS-18 followed by FGS-16, when the results are compared with pentamidine isethionate (Figure 6). It was also observed that the derivatives FGS-16 and FGS-18 showed better target interaction energy over the eight replicates than FGS-3, which is one of the precursors of the derivatives, according to Table 2. In addition, these derivatives showed a tendency to hydrogen bond formation, leaving the interaction of the target (2VPM) and molecules more stable (Figure 7).

Table 2. Interaction energy of bioactives with targets, triphathion reductase (PDB:2WP5) and triphathion synthetase (2VPM)

Samples	Pentamidine isethionate	ACD43	ACD58	CNFD	AGD	AMR	NRG
	Kcal/mol						
2WP5	-8.335±0.10	-10.060±0,15	-9.240±0.20	-9.581±0.21	-4.724±0.13	-10.420±0.15	-7.468±1.13
2VPM	-6.055±0.12	-10.120±0.10	-10.440±0.16	-8.062±0.20	-4.744±0.12	-9.885±0.16	-8.138±0.10

Number of replicates = 8.

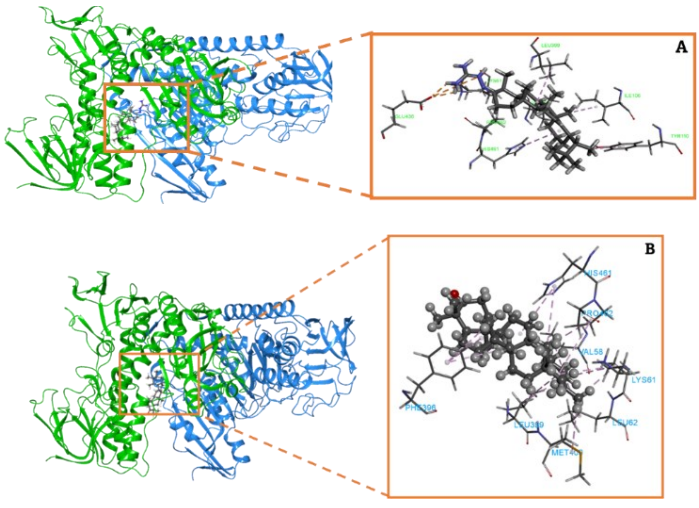

Figure 6. Interaction of 2WP5 with the derivatives of greatest affinity FGS-16 (A) and FGS-3 (B).

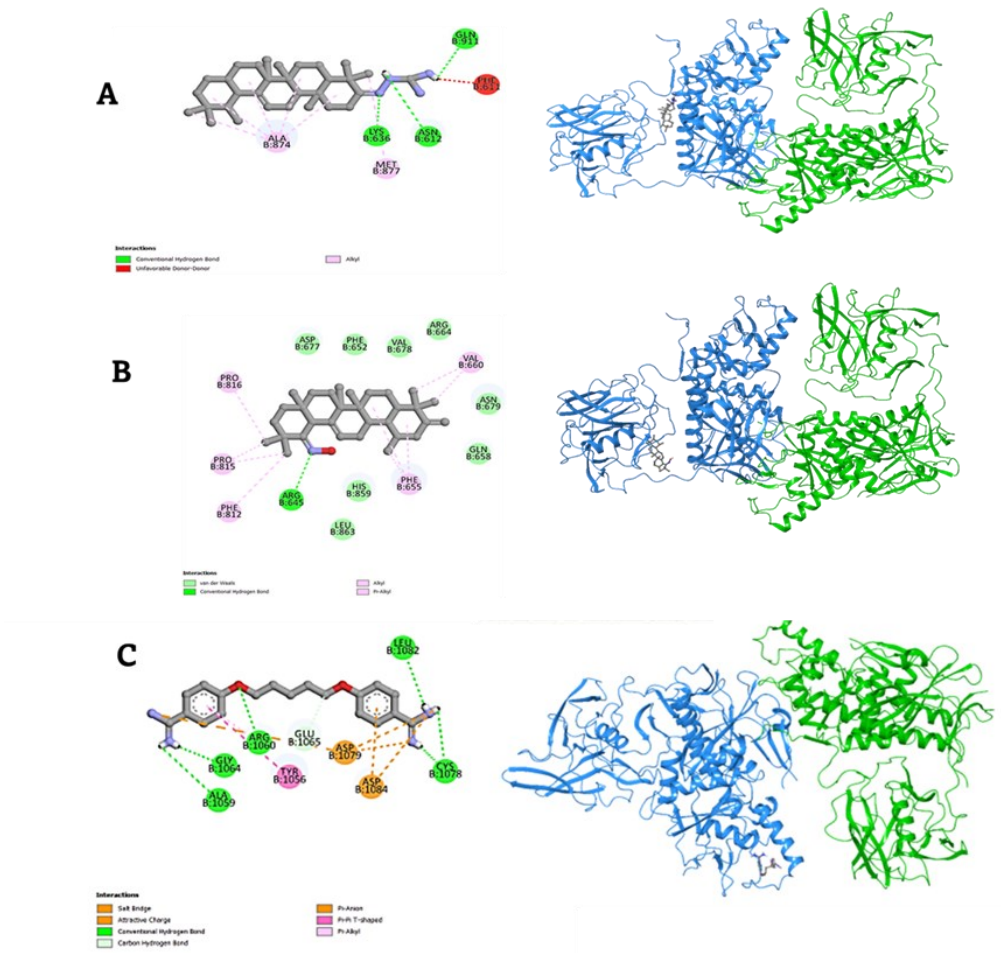

Figure 7. Representations of surfaces with tendencies to hydrogen bonds and interaction of 2VPM with the derivatives FGS-16 (A), FGS-3 (B) and pentamidine isethionate (C) of greatest affinity.

The result of re-docking for the alignment of the 3,4-dihydroquinazoline inhibitor of the enzyme triphathione reductase (PDB ID: 2WP5) was 0.9688 Å, which is within the acceptable value for re-docking and which cannot exceed the range between 1.5–2.0 Å of difference between experimental and computational conformation [22].

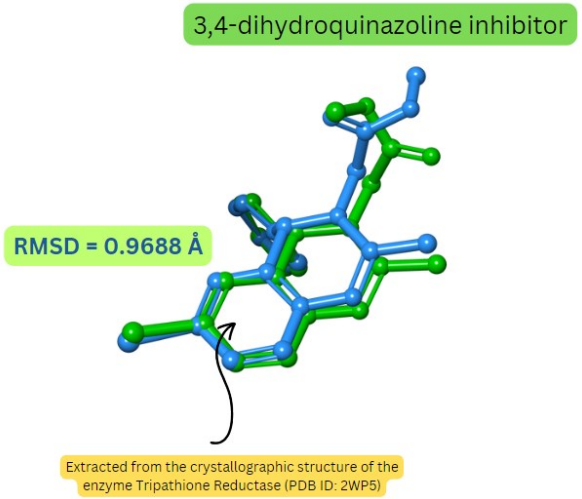

Figure 8. Structural alignment of the inhibitor 3,4-dihydroquinazoline in the enzyme triphathione reductase (PDB ID: 2WP5). In green, the experimental conformation is observed and, in blue, the one obtained by re-docking.

4. DISCUSSION

From the viability tests with the J774 macrophages, the molecule 6b,7-dihydro-5H-cyclopenta[b]naphtho[2,1-d]furan-5,6(9aH)-dione (CNFD) showed a CC_{50} of 2.4 ± 0.8 μg.mL^{-1} at 72 h, which is considered a toxic profile, a result also seen in the study of De Almeida et al. (2021) [21] in human fibroblast cells (MRC5) with a CC_{50} of 3.76 μM. The molecules aminoguanidine, amyrone and naringenin showed a moderate cellular toxicity in this study. However, the results observed in this study were not similar to the study of De Souza et al. (2024) [23], which, in THP1 cells, showed a viability greater than 1,000 μg.mL^{-1} for aminoguanidine and 800 μg.mL^{-1} for naringenin at 48 h. As for hydroxylamine, the test was not performed; however, in the literature, an IC_{50} value of 296.6 μg.mL^{-1} for hydroxylamine is indicated for adherent epithelial cells of monkey kidney (Vero) [24]. Amyrone showed a CC_{50} of 236.1 μg.mL^{-1} in J774 cells. Similar findings were found in the study of De Almeida et al. (2015) [25] but with a cytotoxic effect of triterpene α, β-amyrone of IC_{50} > 20 μg/mL in J774 macrophages. The study of Cota et al. (2011) [26] demonstrated the absence of toxicity against human leukocytes after 48 h of incubation at a concentration of 20 μg/mL of β-amyrone. The study of Guilhon-Simplicio et al. (2022) [12] showed the cytotoxicity of triterpene derivatives in human fibroblasts and murine macrophages. O derivado 16a/16b mostrou uma elevada toxicidade para MRC-5 de IC_{50} 5.19 μM. In our study, the derivative FGS-16 (Urs- and olean-12-ene 3β-guanidine [3-guanidine α- and β-amyrin]) demonstrated a CC_{50} = 32 ± 0.4 μg.mL^{-1} at 72 h in J774 macrophages.

Regarding the antileishmanial activity, aminoguanidine showed an IC_{50} of 62.2 ± 2.5 μg.mL^{-1} for *Leishmania guyanensis* and an IC_{50} of 82.2 μg.mL^{-1} against *Leishmania amazonensis* at 72 h. It did not show inhibition of promastigote forms; however, previous studies report a potential leishmanicidal effect [27]. Amyrone showed an IC_{50} of 236.1 ± 1.5 μg.mL^{-1} for *L. guyanensis* and an IC_{50} of 39.1 ± 2.5 μg.mL^{-1} against *L. amazonensis* during the same period, thus showing a low antileishmanial activity for both species.

Regarding the derivative, FGS-16 (amyrenone plus aminoguanidine) presented an IC_{50} of 0.7 ± 0.2 μg.mL^{-1} for the inhibition of promastigote forms of *L. guyanensis* and 0.5 ± 0.6 μg.mL^{-1} against *L. amazonensis* at 72 h, showing a promising antileishmanial potential for the two species studied. Likewise, it showed an IC_{50} = 3.56 μM against *Leishmania infantum* [12], thus corroborating our study. It is noteworthy that the molecules that served as the basis for the chemical reaction of this derivative did not show good biological activity in inhibiting the promastigotes of the species. In your turn, FGS-18 (3-oxime-urs- and 3-oxime-olean-12-ene [3-oxime α- and β-amyrin]) from the chemical reaction between amyrone plus hydroxylamine showed a low antileishmanial activity at 72 h, with an IC_{50} of 47.3 ± 0.2 μg.mL^{-1} for promastigote forms of *L. guyanensis* and 39.6 ± 0.3 μg.mL^{-1} against *L. amazonensis*.

In the study of Guilhon-Simplicio et al. (2022) [12], the derivative 14a/14b (3-oxime-urs- and 3-oxime-olean-12-ene [3-oxime α- and β-amyrin]) was active against *T. cruzi* and *C. albicans* at the nanomolar scale (2.00 and 2.49 nM, respectively) after being complexed with cyclodextrins to improve its solubility. Thus, it is possible to suggest that inclusion complexes may actually have an important impact on the potency and selectivity of these derivatives, which is already being investigated by the research group. In another study with ninety aminoguanidine hydrazone derivatives evaluated against amastigotes of *L. infantum chagasi*, seven showed leishmanicidal activity of over 50% and an IC_{50} of less than 10 μM [28].

As for the substance, 6b, 7-dihydro-5H-cyclopenta [b] naphtho [2,1-d] furan-5,6 (9aH) -dione (CNFD) inhibited the promastigote forms of *L. guyanensis* with an IC_{50} of 17 ± 5.4 μg.mL^{-1} and 1.2 ± 2.3 μg.mL^{-1} for *L. amazonensis*, showed promise for both species, but needs to be further investigated, as a study has shown it to be a toxic molecule for the human fibroblast lineage MRC-5 with a CC_{50} of 3.76 μM (3.38 – 4.18) in 72 h [21]. Naringenin (NRG) showed an IC_{50} of 65.9 ± 5.2 μg.mL^{-1} at 72 h in the inhibition of *L. guyanensis*. For the inhibition of *L. amazonensis*, NRG showed an IC_{50} of 37.5 ± 4.1 μg.mL^{-1} at 72 h. This molecule showed high activity against *Leishmania*. However, one study showed an IC_{50} of 5 μg.mL^{-1} for narigenin in inhibiting the growth of amastigote forms of *L. donovani*, *in vitro* and *in vivo*. The study also demonstrated moderate leishmanicidal activity for the promastigote forms of *L. amazonensis* and *L. chagasi*. Still, it is worth mentioning that, due to the particularity of propolis regarding its chemical composition, its activity will depend on biotic and abiotic factors of the region where it was

collected, and these leishmanicidal results and the mechanism of action are evidence found in bioactive propolis collected in Bahia, Brazil [29]. The study of Greecco et al. (2012) [30] tested this bioactive after isolating it from *Baccharis retusa*, though it did not show antileishmanial activity. Therefore, the antileishmanial potency of the isolated bioactive (NRG), seems to depend on the region where the plant is collected.

After obtaining the selectivity index (SI), the derivative FGS-16, against *L. guyanensis* and *L. amazonensis*, showed a higher antileishmanial potential in relation to the other molecules, and by means of a mathematical calculation, CC_{50}/IC_{50}, an SI= 45.7 and 64, respectively, thus showing a safe toxic profile. Although the FGS-18 derivative did not show antipromastigote potential, it showed a wide cellular toxicological safety, obtaining an SI= 8.3 for *L. amazonensis*, when compared to pentamidine isethionate which has an SI= 0.3 for the same species. These results are pioneering, as the derivatives are novel for antileishmanial activity against the cutaneous form.

The use of bioactives extracted from natural products may present disadvantages due to the low concentration of the selected substances, which often makes commercial exploitation unfeasible. However, the synthesis of these substances and planned derivatives allows the establishment of the pharmacophoric group and modulation of the biological profile, for example: the molecule [2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone] (LQB-118) acted in different clinical forms of leishmaniasis, with no signs of toxicity at therapeutic doses [31]. However, of the series of twenty pterocarpanquinones tested in *L. amazonensis*, only 10 % of the synthesized molecules presented $IC_{50} > 10\mu M$ on promastigotes, with low toxicity to murine lymphocytes [32]. As for the synthesized hydrazones, the compounds HDZ-3 and HDZ-5 showed relevant activity against promastigote forms of *L. amazonensis*, while HDZ-4 showed better selectivity [33]. Another in vitro study showed a potent leishmanicidal effect on promastigotes of *L. infantum* and *L. braziliensis* strains, with a low cytotoxic effect on murine macrophages and a high selectivity index [34]. These results corroborate our findings.

The molecular docking analysis suggests that the active ingredients that showed the best interactions with the active site of trypanothione reductase (2WP5) of *Leishmania infantum* were the molecules FGS-16 and FGS-3, when compared with the results of the reference drug, pentamidine isethionate. Since the identification of the enzyme trypanothione reductase (TR), research has addressed the search for inhibitors in order to interfere in the life cycle of these flagellates. Thus, studies have reported the inhibitory capacity of TR via the drug clomipramine, which is registered as a classic tricyclic antidepressant. Initially, it was related to an imbalance in the electrochemical gradient of the membranes of parasites; however, later work carried out with a recombinant TR and *in silico* assays indicated its connection with the inhibition of this enzyme [35].

Trypanothione reductase is a homodimer and each subunit of this enzyme has 491 amino acids comprising three different domains: a FAD binding domain (residues 1-160; 289-360), a NADPH binding domain (residues 161-288) and an interface domain (residues 361- 488) [36]. Therefore, it is the main enzyme of the defense system against oxidative stress in trypanosomatids and is absent in mammalian cells. This system is based on the reduction of reactive oxygen species levels, contributing to the maintenance of a reducing intracellular environment. Redox homeostasis is maintained through the conversion of oxidized trypanothione (T(S)2) into reduced trypanothione (T(SH)2) via the enzyme trypanothione reductase, with NADPH as a cofactor [37].

In addition to the role of TR in controlling the redox balance in trypanosomatids, it has been shown that this enzyme can be an important factor of viability and virulence in *Leishmania donovani*. Dumas et al. demonstrated that the knockout of trypanothione reductase of *L. donovani* significantly decreased the infectivity of these parasites and their ability to survive inside macrophages [38].

In the study by Tovar et al. (1998) [39], the authors demonstrated that the substitution of the trypanothione reductase coding sequence drastically affected the ability of *Leishmania donovani* parasites to survive within cytokine-activated macrophages, showing that TR is an essential enzyme for the survival of these parasites.

As for the target trypanothione synthetase (2VPM), the best interaction was shown by FGS-18 followed by FGS-16. It was also observed that the derivatives FGS-16 and FGS-18 showed better target interaction energy over the eight repetitions than FGS-3, one of the

precursors of the derivatives. In addition, these derivatives showed a tendency to hydrogen bond formation, leaving the target interaction (2VPM) and molecule more stable.

Trypanothione is synthesized by an ATP-dependent monomeric ligase, trypanothione synthetase (TryS), in a two-step reaction. In the first, there is the junction of a molecule of GSH and one of Spd, forming the intermediate product, glutathionylspermidine. The second step consists of binding the intermediate of the first reaction, glutathionylspermidine, with one more glutathione molecule. TryS also has an amidase domain that catalyzes the hydrolysis of T(SH)2 in GSH and Spd, and is important to maintain the homeostasis of T(SH)2 [40].

Trypanothione is the most powerful reducing agent in trypanosomatids when compared to the other thiols available (glutathionylspermidine, GSH and ovothiol) [41]. This molecule is oxidized and returns to its reduced form by the action of the flavoenzyme trypanothione reductase, which uses an NADPH molecule in the process [42]. Trypanothione is the most powerful reducing agent in trypanosomatids when compared to other available thiols such as glutathionylspermidine, GSH and ovothiol [41]. Therefore, this enzyme is unique in trypanosomatids, having no corresponding gene sequence in the mammalian host genome [43]. The TryS gene is located on chromosome 27. The coding sequence of this gene for *L. braziliensis* M2904 (LbrM.27.2010) presents 1959 bp and 652 amino acids in its predicted protein sequence, giving rise to a protein of approximately 74 kDa (tritrypdb.org). Thus, all the characteristics of TryS, i.e., being present only in trypanosomatids; being encoded by a single gene in the genome; being essential; being expressed in low abundance and having no alternative by-pass, make TryS one of the best targets for drug development for the trypanothione biosynthesis pathway [43].

It is interesting to note that, in molecular docking analyses, Guilhon-Simplicio et al. (2022) [12] also observed good interaction and complementarity of FGS-16 and FGS-18 with the active site of CYP51, an important enzyme involved in the synthesis of ergosterol in the membrane of parasites.

The study demonstrated that the CNFD derivative presented interesting results against both *Leishmania* species, but showed cellular toxicity. The FGS-18 derivative, on the other hand, showed low antileishmanial activity in promastigotes. Despite the limitations of these derivatives, an alternative to improve the biological action of these molecules would be to incorporate them into nanotechnological carriers that can enable greater solubility, permeability, delivery of the active ingredient to the site of action, among others.

5. CONCLUSION

Through *in vitro* and *in silico* investigations, the FGS-16 derivative was shown to be non-toxic in cells and showed promising antileishmanial activity against promastigotes, in addition to presenting three hydrogen bonds and better couplings in the molecular docking of the 2VPM and 2WPS targets. Therefore, it is a candidate for future preclinical and clinical phases.

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