CAPÍTULO 12

ANTI-LEISHMANIA ACTIVITY OF FRAXETIN INDUCES CHANGES IN PROTEOME PROFILE IN LEISHMANIA INFANTUM SPECIES

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Klewdma De Freitas Araújo
Universidade Federal de Pernambuco, Programa de Pós-Graduação em Inovação Terapêutica, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0009-0008-7792-7521

Paloma Lys De Medeiros
Universidade Federal de Pernambuco, Departamento de Histologia e Embriologia, Laboratório de Cultura de Tecidos, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0001-9517-1416

Fabiana Aparecida Cavalcante Silva
Centro de Tecnologias Estratégicas do Nordeste (CETENE), Laboratório de Diagnose Fitossanitária e Fidelidade Genética, Av. Prof. Luís Freire, 1 - Cidade Universitária, 50740-545 – Recife – PE, Brasil.
https://orcid.org/0000-0002-4674-895X

Elton Pedro Nunes Pena
Universidade Federal de Pernambuco, Departamento de Genética, Laboratório de Genômica e Proteômica de Plantas, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0002-6822-2123

Gustavo Barbosa De Lima
Instituto Aggeu Magalhães / Fiocruz-PE, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50740-465 – Recife – PE, Brasil.
Laboratório de Imunopatologia Keizo Azami, Setor de Microscopia Eletrônica, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0009-0000-7000-2026

Fábio André Brayner Dos Santos
Instituto Aggeu Magalhães / Fiocruz-PE, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50740-465 – Recife – PE, Brasil.
Laboratório de Imunopatologia Keizo Azami, Setor de Microscopia Eletrônica, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0002-8017-6780

Gabriel Gazzoni Araújo Gonçalves
Instituto Aggeu Magalhães / Fiocruz-PE, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50740-465 – Recife – PE, Brasil.
Laboratório de Imunopatologia Keizo Azami, Setor de Microscopia Eletrônica, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0001-7723-3134

Luiz Carlos Alves
Instituto Aggeu Magalhães / Fiocruz-PE, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50740-465 – Recife – PE, Brasil.
Laboratório de Imunopatologia Keizo Azami, Setor de Microscopia Eletrônica, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0002-8384-3008

João Soares Brito Da Luz
Universidade Federal de Pernambuco, Hospital das Clínicas, Av. Prof. Moraes Rego, 1235 – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0001-6950-8484

Erwelly Barros De Oliveira
Universidade Federal de Pernambuco, Departamento de Antibióticos, Laboratório de Cultura de Células, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0001-9581-0791

Tercílio Calsa Junior
Universidade Federal de Pernambuco, Departamento de Genética, Laboratório de Genômica e Proteômica de Plantas, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0002-8302-2031

Dijanah Cota Machado
Universidade Federal de Pernambuco, Departamento de Biofísica e Radiobiologia, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0002-5466-0229

Claudio Gabriel Rodrigues
Universidade Federal de Pernambuco, Departamento de Biofísica e Radiobiologia, Av. Prof. Moraes Rego, S/N – Cidade Universitária, CEP: 50670-901 – Recife – PE, Brasil.
https://orcid.org/0000-0001-9015-5644

ABSTRACT: The goal of this study was to evaluate the anti-leishmania activity of fraxetin (6-methoxy-7,8-dihydroxycoumarin) against a strain of *Leishmania infantum* and to identify possible alterations in the parasite's proteome. Promastigotes forms of *L. infantum* (2×10^6 parasites/mL) were tested with fraxetin at different concentrations (0.195 to 100 μg/mL). Promastigotes were sensitive to fraxetin and we obtained an IC_{50} of 6.25 μg/mL. To evaluate the effect of the fraxetin, proteomic analysis was performed by LC-MS/MS that identified a total

of 1,565 proteins, of which 359 had differences in accumulation after treatment with fraxetin. In particular, 144 proteins were overaccumulated and 215 proteins were downregulated, which could be the result of drug effects on the *Leishmania* gene expression. For the integration of experimental data, the BlastGO program was used for genetic ontology. Due to the action of fraxetin, the flagellar component of *Leishmania* seemed to have been affected. According to putative data, 12 proteins involved in the regulation of this important structure were inhibited. Scanning electron microscopy of promastigotes treated with fraxetin showed parasitic aggregations, rounder form with shortened flagella and indicating functional and structural abnormalities. Further studies are needed to consider fraxetin as an anti-leishmania candidate.
KEYWORDS: Anti-leishmania activity, Fraxetin, *Leishmania infantum*, Leishmaniasis, Natural products, Proteomics.

INTRODUCTION

Leishmaniasis is a tropical disease found in more than 100 countries. In Africa, Southeast Asia and Americas, this disease occurs endemically with high infection rates (Bilgic-Temel et al. 2019). The World Health Organization estimates the emergence of 700,000 to 1 million new cases and 20,000 to 30,000 deaths every year (Tabbabi 2019, World Health Organization 2020). Leishmaniasis is caused by an obligate intracellular parasite of the genus *Leishmania* that harbors several species responsible for different clinical forms (Hilaire et al. 2022).

Leishmania species are unicellular eukaryotes having a well-defined nucleus and other cell organelles including kinetoplasts and flagela (Ilıkçı Sağkan et al. 2022). The most serious clinical form is the visceral, characterized by the reproduction of parasites inside the cells of the mononuclear phagocyte system, affecting the liver and causing morphofunctional changes with consequences for the functioning of the whole organism (Kaufer et al. 2017, Moriconi M. et al. 2017, Varela et al. 2021).

Some species of *Leishmania* are resistant to currently used chemotherapy drugs, making treatment difficult, contributing to leishmaniasis as the third-most lethal neglected disease, despite prevention and control efforts set by the Board of Directors of the Pan American Health Organization. Health/World Health Organization (PAHO/WHO) (Maia-Elkhoury et al. 2020, Barreto et al. 2022). In this regard, the improvement of studies of biologically active molecules in promising plant species is an important direction in the search for new herbal remedies (Soosaraei et al. 2017). Several studies based on natural compounds to combat leishmaniasis have already been developed (Gervazoni et al. 2020, Sakyi et al. 2021), highlighting the study by Peixoto et al. (2021) evaluating the use of *Piper spp.* against different species of *Leishmania* that cause cutaneous and visceral forms.

Our group was studying the anti-leishmania potential of fraxetin (7,8-dihydroxy-6-methoxycoumarin), a secondary metabolite present in higher plants with already established

pharmacological, biochemical and therapeutic activities (Thuong et al. 2009, Wang et al. 2014, Song et al. 2021). In this work we aimed to evaluate fraxetin in an initial analysis regarding its anti-leishmania potential through a screening test and identify possible molecular mechanisms involved through proteomic analysis.

MATERIAL AND METHODS

Test compound

Fraxetin (M.W. 208.17) was provided by Professor José Maria Barbosa Filho (Laboratory of Pharmaceutical Technology, Federal University of Paraíba).

Cultivation of the Promastigotes Forms of *L. Infantum*

Promastigote forms of *L. infantum* (MHOM/BR2000/Merivaldo2) were provided by Dr. Osvaldo Pompílio de Melo Neto from the Microbiology Department of the Aggeu Magalhães/FIOCRUZ Research Center. They were maintained in LIT (Liver Infusion Broth) medium supplemented with 20% FBS (Fetal Bovine Serum) (LGC Biotechnology Ltda.), 0.2% hemin (Sigma-Aldrich) and 0.1% antibiotics (penicillin-streptomycin) (Sigma-Aldrich) in a BOD incubator at 26 °C.

Evaluation of anti-leishmania Activity by the MTT method

To determine the IC_{50}, tests were carried out in triplicate using the MTT colorimetric method (MOSMAN 1983), through which the percentage of reduction in cell viability was analyzed. Promastigote forms in the exponential growth phase were treated with fraxetine at concentrations between 100 and 0.195 μg/ml and distributed in 96-well plates, at a concentration of 2×10^6 cells/mL of LIT medium. As a positive control, it was used Amphotericin B (Sigma-Aldrich) and LIT medium as a negative control. The plates were incubated in a BOD incubator at 26 °C for 72 hours and then 20 μL of MTT (Sigma-Aldrich) at a concentration of 5 mg/mL was added to each well and the plates were subjected to a new incubation for 4 hours. Subsequently, 100 μL of DMSO was added for the solubilization of the formazan crystals of and the absorbance was measured in a spectrophotometer at 585 nm.

Proteomic analysis

Protein extract preparation

The experiments were carried out with parasites in the exponential growth phase and

divided into two groups: 1) control group free of treatment with fraxetin; and 2) group treated with this substance at the IC_{50} concentration. Three cultures from each group were obtained, centrifuged, washed with PBS, lysed by liquid nitrogen maceration method and stored at -20 °C for protein extract preparation. Protein extraction was performed using the trichloroacetic acid (TCA) precipitation protocol associated with acetone (Damerval et al. 1986) combined with protein solubilization by the urea-thiourea method (Rabilloud et al. 1997).

Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were quantified by Bradford method (1976). Then, 20 μL of the final volume of these samples were applied in quintuplicate on a 13% SDS-PAGE electrophoresis, subjected to 30 mA for 35 minutes. At the end of the run, the gel was stained with Coomassie Blue (R-250) 0.1% for 20 minutes and then immersed overnight in bleaching solution (30% methanol, 10% acetic acid, 60% water), with subsequent cutting of the gel to separate the protein bands, which were sent to analysis by LC-MS/MS.

Liquid Chromatography coupled to Sequential Mass Spectrometry (LC-MS/MS)

The LC-MS/MS technique used two integrated systems: liquid chromatograph nano LC-1D Plus and Autosampler as-2 (Eksigent) containing a column 15 cm long with 75 mm internal diameter containing C18 particles of 3 micrometers of diameter coupled to an LTQ Orbitrap XL ETD hybrid mass spectrometer (Thermo Scientific). For chromatography, two liquid gradients were used: 0.1% formic acid, 5% DMSO in phase A; and 0.1% formic acid, 5% DMSO in acetonitrile in phase B, injected through a binary high pressure pump (channel A and channel B) at a flow rate of 250 nL/min and linear gradient from 5 to 40% of phase B in 120 min. The samples were introduced into the spectrometer through a nanoelectrospray probe which is the ionizing source, the scanning mass range was 300-2,000 (*m/z*) with a resolution of 60,000 m/z, the source voltage was 2.70 kV and the current 100 **μ**A. Sample processing was carried out using the MaxQuant software (version 1.5.8.3) using the Andromeda algorithm to search for proteins in the Uniprot database, using as parameters: methionine oxidation (variable modification) and cysteine carbamidomethylation (static modification); trypsin enzyme; 2 cleavages allowed; mass range in the range 200-2,000; 10 ppm peptide tolerance; 1 Da fragment ion tolerance; minimum 1 unique peptide per protein. The identifications were filtered by the 1% false discovery rate (FDR) at the protein level to remove potential contaminants and analyzed using the Perseus software that identified and quantified the analytes present in the sample.

Scanning electron microscopy

Promastigote forms of *L. infantum* from the groups treated with fraxetine (at the IC_{50} concentration) and control (without treatment) were cultivated for 72 hours in BOD, then

centrifuged at 1,500 *xg* for 10 minutes. After discarding the supernatant, the precipitate was washed 3 times with the addition of 2 mL of 0.1 M phosphate buffer (pH 7.2).

The samples were fixed (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 12 hours at room temperature, washed 3 times every 10 minutes in the same buffer and post-fixed in 1% osmium tetroxide (OsO4) in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 hour. The material was washed three times every 10 minutes in the same buffer, dehydrated in increasing series of ethanol (30, 50, 70, 90 and 100%) and later the samples were sent to the critical point. After mounting and metallization the material, 50 images from each group were observed in SEM (JEOL - JSM 5600LV).

Statistical analysis

Anti-leishmania activity (IC_{50})

The IC_{50} was determined by reducing the absorbance by 50% in relation to the negative control containing only cells free of treatment. The calculation of cell viability was performed as a function of the optical density observed in the group containing only LIT medium whose cell viability was 100%. Data were evaluated using the one-way ANOVA test followed by Tukey's post test with a significance level at $p < 0.05$ and presented as mean ± standard deviation from three independent experiments performed in triplicate. Statistical analysis was performed using the OriginPro 8 software (OriginLab Corporation).

Proteomic evaluation

To compare differentially accumulated proteins (expressed and repressed) the Student's t test was used and the results were considered significant at $p < 0.05$.

RESULTS

Anti-leishmania activity

Through the MTT assay, the parasites were subjected to different concentrations of fraxetin (0.195 to 100 μg/mL) to determine which concentration would be observed to reduce cell viability by 50% (IC_{50}). We obtained the IC_{50} in the concentration of 6.25 μg/ml (Figure 1).

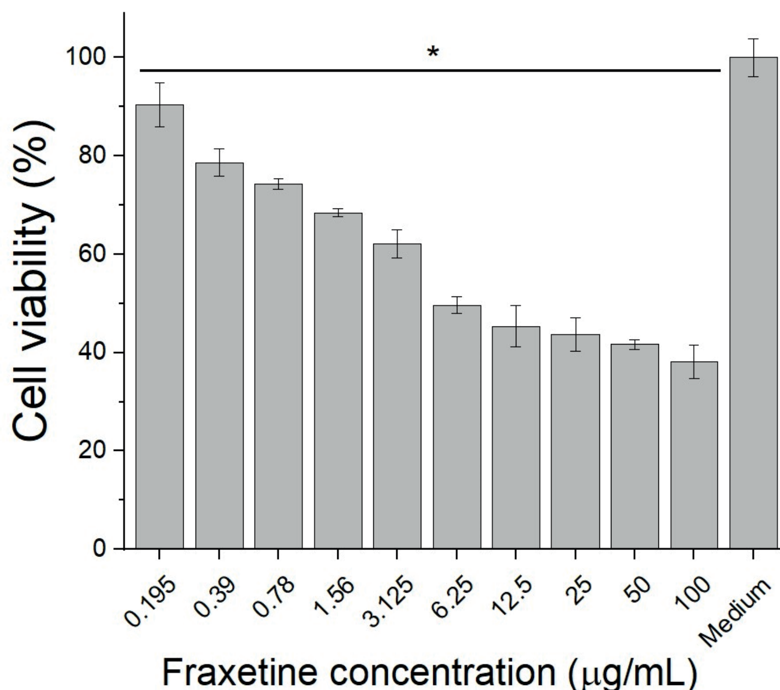

Figure 1. Fraxetin IC_{50} determination. Evaluation of the anti-leishmania activity of fraxetin on promastigotes of *L. infantum*, submitted to concentrations between 0.195 and 100 μg/mL, during 72h of incubation in BOD at 26 °C. Tests were performed in triplicate. Data presented as mean ± SD. * $p < 0,5$ (one-way ANOVA followed by Tukey's post test).

Proteomics

The accumulated proteins profile of promastigotes forms of *L. infantum* free of treatment and treated with fraxetin at the IC_{50} concentration was obtained by the LC-MS/MS technique. We identified a total of 1565 proteins, of these 359 were differentially accumulated between the two groups of parasites, with increased expression observed in 144 proteins and repressed expression in 215 of them (data not shown).

Gene ontology analysis was performed using the BlastGO software, which categorized these proteins into three levels: cellular component, molecular function and biological process according to their main characteristics.

The flagellar component suffered damage due to the action of fraxetin. According to the BlastGO results, twelve proteins involved in the regulation of this structure were inhibited in *L. infantum* (data not shown).

Scanning electron microscopy

Scanning electron microscopy analysis revealed morphological differences between

promastigotes of *L. infantum* treated and not treated with fraxetin (Figure 2).

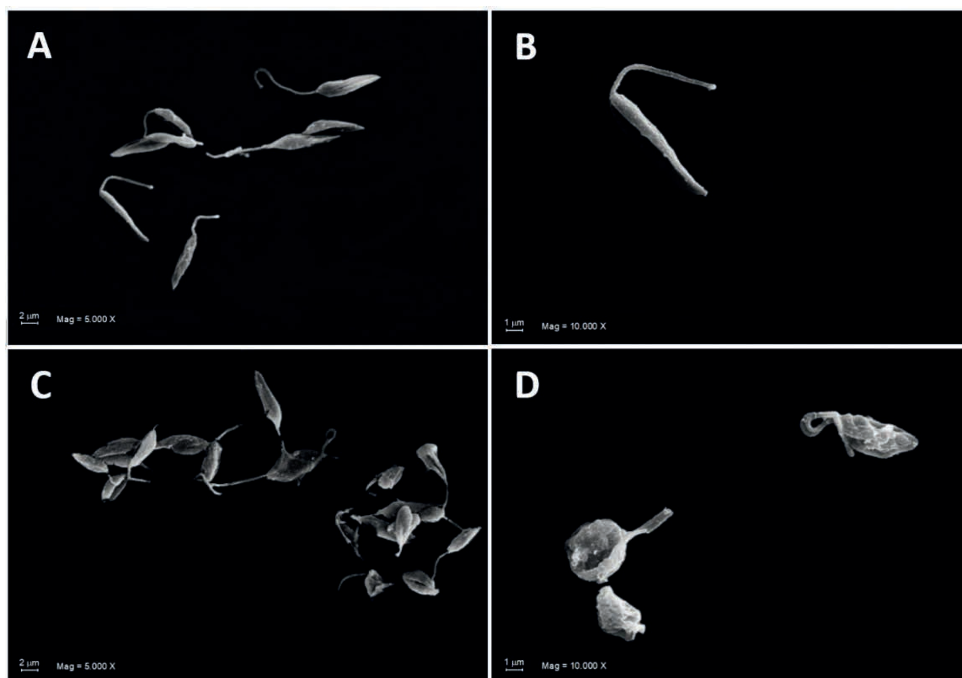

Figure 2. Scanning electron microscopy of promastigotes of *L. infantum* on the action of fraxetin on the IC_{50} concentration for 72 h. (A) and (B) Untreated promastigotes showing isolated cells with regular body morphology and long flagellum. (C) and (D) Treated promastigotes showed alterations, in (C) parasitic clusters with shortened bodies (5,000x magnification); in (D) parasites with short, twisted flagella and wrinkled cell surface (10,000x magnification).

DISCUSSION

In this work, promastigote forms were tested because they provide a good model as an effective and reliable indicator for a primary in vitro evaluation of a molecule with anti-leishmania activity. Experimental trials with promastigote forms also present the requirements for a screening test such as ease of cultivation, good reproducibility and low cost (De Muylder et al. 2011, Siqueira-Neto JL et al. 2010, Sharlow et al. 2009).

Although subsequent trials in other models are necessary in the drug development process, in vitro screening with promastigotes forms that share common metabolic pathways with the intracellular amastigote forms represents a reliable basis for a first indication of whether the test compound acts directly against the parasite or se through activation of effector functions in macrophages (Gebre-Hiwot et al. 1992, Kayser et al. 2001).

Fraxetin is one of the main constituents of the medicinal plant *Fraxinus* sp., where it is involved in the metabolism of iron (Sarfraz et al. 2017). As well as other coumarins, it has multiple bioactivities, such as anticancer, antimicrobial, anti-inflammatory, antioxidant (Qin et al. 2019, An et al. 2020). But its anti-leishmania potential was still unknown until our

work, where we observed *in vitro* experiments a low IC_{50} value (6.25 µg/ml). This IC_{50} value together with the low cytotoxic potential on Vero and HeLa cells (data not shown) encourage the study of fraxetin as a molecule with potential medicinal properties for fighting against lesihmaniasis.

The search for an effective, low-cost, and low-toxicity molecule to combat leishmaniasis has driven research whose targets are natural products derived from plants (Cruz Filho et al. 2023). Some studies already completed highlight the role of coumarins in this field. Tasdemir et al. (2006) investigated the anti-leishmania and cytotoxic activities of different coumarins (scopoletin, 4-hydroxycoumarin, umbelliferone, 4-methylumbelliferone, bergapten, bergaptol and angelicin) against amastigotes forms of *Leishmania donovani* and obtained IC_{50} values of 2.5 µg/ml (bergaptol) and >30 µg/ml (scopoletin and bergapten).

In another study, Kermani et al. (2016) identified the anti-leishmania activity of osthole against amastigotes of *Leishmania major*. It was determined an $IC_{50} \geq 20$ µg/mL and a possible action on the parasite's respiratory chain was suggested as a consequence of the decrease in the production of the enzyme fumarate reductase. Figueiredo Peloso et al. (2020) determined the activity of 8-methoxy-3-(4-nitrobenzoyl)-6-propyl-2H-chromen-2-one against strains of *L. amazonensis* and *L. infantum*, establishing IC_{50} values of 3.2 and 3.8 µM, respectively. The anti-leishmania activity was correlated to the presence of a propyl side chain in the center of the structure linked to a nitro group and, based on these results, the authors suggested that this coumarin compound is very promising for the development of a new anti-leishmania agent.

After IC_{50} determination, we performed a proteomic analysis and scanning electron microscopy of the samples. It is speculated that certain proteins present specific regulation characteristic of the metabolic stage in which the organism finds itself, which helps the parasite to adapt to changing environments. Identifying and targeting these reactions essential to the parasite's survival could therefore lead to the development of better treatment strategies and the eradication of the parasitic infection. Here, we will highlight the role of proteins related to the flagellar component that had a decrease in expression after treatment with fraxetin.

The structural integrity of the parasitic membranes is essential for their survival. As seen in Figure 2, where promastigotes forms of *L. infantum* treated with fraxetin showed more rounded parasites with shortened flagella and parasitic aggregates, indicating functional and structural impairment. Contrasting with forms without fraxetin treatment, which presented long flagella and isolated parasites, demonstrating that there was no functional and structural impairment. These results corroborate with data found by Mondêgo-Oliveira et al. (2021) and Machado et al. (2021) who reported flagellar atypia in *L. infantum*, when subjected to treatments with anti-leishmania action molecules. In electron microscope images, we found that at 10,000 times magnification, *Leishmania* had a more wrinkled surface, which interferes with the flagella's primary function of sensing, adapting, and surviving in a hostile

environment. According to Beneke et al. (2019), disturbance of the flagellar membrane is also capable of interfering with sensory functions mediated by this structure.

The flagellar component is an important mechanism for parasite survival and virulence, whose formation depends on the addition of tubulin subunits at the distal end of microtubules (Husein et al. 2018). Its formation requires the CTT complex, which consists of two subunits (CCT-α and CCT-β) with the function of contributing to the correct folding of cytoskeletal proteins in all eukaryotes (Vallin & Grantham 2019). Misfolded proteins do not perform their functions correctly and often induce dysregulations so intense that they can impair cell survival (Grantham 2020). Structurally, the complex consists of eight individual protein subunits that assemble to form a double-ring barrel structure essential for the correct folding of newly synthesized actin and tubulin through interaction with a co-transcriptional activator. And, in the presence of ATP, two subunits of the CCT-α and CCT-β complex of the actin and tubulin filaments, respectively, form microtubules (Grantham 2020).

We have identified a block in the expression of two constituent proteins of the CCT complex (LINF_320040200 and LINF_270019000), which resulted in impacts on the visible flagellar structure. This statement is confirmed by other works. Lundin et al. (2008) found changes in microtubule-mediated processes associated with reduced CCT levels in *Caenorhabditis elegans*. Saegusa et al. (2014) found alterations in the apical plasma membrane structure in intestinal cell microvilli and actin aggregates. Thus, we state that CCT is essential for the correct functioning of any cellular mechanism that depends on functional microtubules of *Leishmania*.

The maintenance of cell membrane integrity goes beyond the synthesis of cytoskeletal constituents. Several other proteins participate in the regulation of this mechanism, including the G proteins, which are a group of small GTPases grouped into five families that act in the regulation of a wide variety of cells metabolism and are essential in the regulation of intracellular and membrane traffic, which confers stability to the structure (Husein et al. 2018, Maheshwari et al. 2018). These GTPases exert their functions after binding with GTP/GDP inducing an active/inactive state respectively that recruit anchoring factors for the membranes (Sahin et al. 2008).

Cuvillier et al. (2003) demonstrated the role of small G proteins similar to the ADP-ribosylating factor when associated with tubulin folding proteins in microtubule biogenesis and cytoskeletal stability. Lindemann & Lesich (2010) ratified the need for the presence of ADP as an intermediary of the flagellar driving force. While Chauhan et al. (2020) found in their study that mutations in Rab that blocked the connection to GTP/GDP induced alterations or inhibition of intracellular transport. These findings corroborate our study where we verified the decrease in the expression of proteins belonging to the small G protein family (ARL-1, LINF_310031000 and LINF_270013800) altering the traffic intracellularly and inducing instability in tubulin, which consequently weakens the flagellar structure.

One of the most important post-translational modifications in the regulation

of signaling processes is the phosphorylation of proteins, promoted by kinases and phosphatases that play an antagonistic role, respectively with the addition/removal of the phosphate groups of phosphorylated amino acids, especially serine, threonine and tyrosine (Szöör 2010). Phosphorylation induces conformational changes that lead to the formation of protein complexes with new functionalities in cell cycle regulatory signaling pathways, gene transcription, mRNA translation, transport activities and cell motility (Szöör 2010).

Protein phosphatases show great structural and functional diversity, are classified into four main groups, with the serine/threonine group being responsible for most of the dephosphorylation events (Szöör 2010). The study conducted by Escalona-Montaño et al. (2021) related a Serine/Threonine Phosphatase present in *L. major* with the flagellar component, suggesting a possible regulatory activity on this organelle. These findings are supported by our work, in which we also identified serine phosphatase (LINF_280012000), a flagellar component regulator inhibited by fraxetin. In general, proteins are versatile and have multifunctionality participating in the regulation of various cellular processes, closely related or not.

The tetratricopeptide repeat domain of the protein has previously been shown to have a strong influence on periplasmic flagella assembly, morphology, and motility of the Lyme disease spirochete *Borrelia burgdorferi* (Moon et al. 2018). In our work, we revealed inhibition of the tetratricopeptide repeat domain protein (LINF_040009200), which may also be associated with flagellar dysregulation in leishmania under the action of fraxetin. Deficiency in its expression alters the flow of charges, impacting the stability of the flagellar structure (Bürgi et al. 2021).

Nucleoside diphosphate kinase (Ndpk) participates in the assembly and motility of microtubules when associated with tubulin (Miranda et al. 2021). The results of the study by Paul et al. (2014) showed that this protein in *L. major* has an electronegative potential of the active site greater than the homologue in the human species. This leads to the safe development of a drug that seeks to inhibit this protein, since the human protein would not be affected, thus avoiding the appearance of adverse reactions.

Vieira et al. (2017) studied the action of the SU11652 molecule, a Ndpk inhibitor, on different strains of Leishmania, with results observed on *L. infantum*. It was observed that the parasite was insensitive to SU11652 or Ndpk did not play a key role in the survival of this species. Thus, the existence of Ndpk inhibitors with anti-leishmania activity is unknown, however, our work identified the negative regulation of this protein, raising the possibility that this can be a path in the development of a molecule with anti-leishmania activity that does not induce the development of side effects in people with leishmaniasis.

We also observed suppression of aspartate carbamoyltransferase (LINF_160010500), responsible for promoting the condensation of carbamoyl-phosphate and L -aspartate to form N-carbamoyl-L-aspartate and phosphate, as well as being involved in *de novo* pyrimidine biosynthesis. Pyrimidine nucleotides also function as intermediate metabolites

in various cellular processes including the synthesis of membrane phospholipids, impacting the maintenance of the parasite's membrane integrity (Lunev et al. 2018, Bosch et al. 2020, Li et al. 2021).

Protein-protein interactions are essential in regulating biological processes and maintaining cellular homeostasis (Stevers et al. 2018, Obsilova & Obsil 2020). The 14-3-3 protein family is responsible for several protein interactions, contributing to the export or blocking of signals or functioning as adapter proteins linking two phosphorylated proteins and thus helping to regulate a huge variety of processes: signal transduction, cell cycle, apoptosis, aids in the folding of other proteins and intracellular cargo trafficking, an important mechanism for flagellar stability and functionality. We identified the suppression of two 14-3-3 proteins (LINF_110008400 and LINF_360040400), which impacted the stability of microtubules. This finding is confirmed by the study conducted by Stevers et al. (2018) who observed that in *Plasmodium* sp., this protein class interacts with host cells, inducing changes that influence the remodeling of the erythrocyte cytoskeleton. That is, this protein could change the molecular structure of its binding partners due to its rigid α-helical structure that forces conformational changes in other proteins (Pennington et al. 2018).

Thus, we emphasize that fraxetin induced severe flagellar damage, altering assembly, driving force and stability, modifying both the morphology and functionality of the structure of *Leishmania infantum*.

Our results revealed that fraxetin exhibited anti-leishmania activity and was able to induce alterations in the flagellum, an important cellular component responsible for mobility and which also plays an important role in the perpetuation of the species in nature, because besides to perceiving hostile changes in the microenvironment and transmitting information for metabolic adaptations, it is through this structure that the membrane invaginations necessary for the morphological changes of the different stages of the parasite's life cycle to occur between vertebrate and invertebrate hosts. Despite the promising results, additional future studies are needed for this molecule to be considered eligible for the development of an effective and safe therapeutic candidate against leishmaniasis.

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