UNIVERSIDADE FEDERAL DO PARANÁ

VINÍCIUS LOPES LESSA

AVALIAÇÃO *IN VITRO* DO EFEITO COMBINATÓRIO DE NARINGENINA COM MILTEFOSINA CONTRA *Leishmania amazonensis*

CURITIBA

2025

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AVALIAÇÃO *IN VITRO* DO EFEITO COMBINATÓRIO DE NARINGENINA COM MILTEFOSINA CONTRA *Leishmania amazonensis*

Tese apresentada ao curso de Pós-Graduação em ciências veterinárias, Setor de Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em ciências veterinárias.

Orientador(a): Prof(a). Dr(a). Rafael Felipe da Costa Vieira

Coorientador(a): Prof(a). Dr(a). Fabiano Borges Figueiredo

CURITIBA

2025

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
UNIVERSIDADE FEDERAL DO PARANÁ
SISTEMA DE BIBLIOTECAS – BIBLIOTECA DE CIÊNCIAS AGRÁRIAS

Lessa, Vinícius Lopes
Avaliação *in vitro* do efeito combinatório de naringenina com miltefosina contra *Leishmania amazonensis* / Vinícius Lopes Lessa. – Curitiba, 2025.
1 recurso online: PDF.

Tese (Doutorado) – Universidade Federal do Paraná, Setor de Ciências Agrárias, Programa de Pós-Graduação em Ciências Veterinárias.
Orientador: Prof. Dr. Rafael Felipe da Costa Vieira
Coorientador: Prof. Dr. Fabiano Borges Figueiredo

1. Doença transmissível. 2. Leishmaniose. 3. Leishmaniose cutânea. I. Vieira, Rafael Felipe da Costa. II. Figueiredo, Fabiano Borges. III. Universidade Federal do Paraná. Programa de Pós-Graduação em Ciências Veterinárias. IV. Título.

Bibliotecária: Ana Camila Quaresma Moura CRB-9/2212

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **VINÍCIUS LOPES LESSA**, intitulada: **Avaliação in vitro do efeito combinatório de naringenina com miltefosina contra Leishmania amazonensis**, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 14 de Março de 2025.

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AGRADECIMENTOS

Agradeço primeiramente a Deus pelas experiências desta vida que me possibilitaram crescer e aprender cada vez mais como ser humano.

Agradeço a minha família e em especial aos meus pais, Carlos Roberto Lessa e Jandira Lopes Lessa, por todo apoio emocional e financeiro.

Agradeço a toda equipe do laboratório de vetores da Universidade Federal do Paraná no setor de Ágrárias em especial ao meu orientador o Drº Rafael Felipe da Costa Vieira, por todo apoio financeiro e oportunidade realizar esse doutorado.

Agradeço a toda a equipe do laboratório de biologia celular do Instituto Carlos Chagas (ICC) e em especial pelo meu coorientador o Drº Fabiano Borges Figueiredo, por todo apoio financeiro, emocional e instrumental para a realização desse trabalho.

Agradeço a todos os professores da pós-graduação em ciências veterinárias da UFPR por todo conhecimento passado.

Agradeço aos meus amigos em especial a Isadora Picasky Deip e Bruna Almeida de Jesus por todo apoio emocional, carinho e momentos de alegrias que apenas amizades verdadeiras podem ofertar nessa vida.

Agradeço à coordenação de aperfeiçoamento de Pessoal de Nível Superior e ao conselho nacional de desenvolvimento pela bolsa de Doutorado.

RESUMO

A leishmaniose é uma doença causada pelo protozoário unicelular do gênero Leishmania, sendo a forma cutânea a mais difundida no mundo, sendo uma das espécies a *Leishmania amazonensis* causadora de diferentes formas clínicas, como leishmaniose cutânea localizada (LCL), leishmaniose cutânea disseminada (LD) e leishmaniose cutânea difusa (LCD) no Brasil. Os medicamentos de primeira e segunda escolha mais utilizados no tratamento da leishmaniose apresentam alta toxicidade e baixa eficiência na cura dos pacientes com a forma LCD. Portanto, faz-se necessária a investigação de novas terapias utilizando compostos leishmanicidas menos tóxicos, como os flavonoides, e sua combinação com medicamentos convencionais. Neste estudo os principais objetivos foram primeiro explorar por revisão de literatura a ação *in vitro* de flavonoides extraídos de fontes naturais em *Leishmania amazonensis* além de buscar as principais metodologias empregadas para o estudo destes compostos. Outro objetivo foi combinar a miltefosina ao flavonoide naringenina para investigar um possível efeito sinérgico entre os dois compostos. Os resultados *in vitro* mostraram que a naringenina apresenta concentração inibitória (CI_{50}) de 219,9 μM e miltefosina de 13,21 μM, nas análises *in silico* foi utilizada a regra de cinco de *Linpinski's* para avaliar absorção oral de naringenina, que atende aos cinco critérios utilizados por essa análise qualitativa. Foram realizados ensaios *in vitro* para a construção de isobolograma que resultou nos cálculos de índice de redução de dose (DRI) e índice de concentração inibitória fracionada (FICI) que demonstraram que a combinação de naringenina com miltefosina apresenta efeito aditivo, sendo capaz de reduzir o CI_{50} de miltefosina em aproximadamente duas vezes nas formas promastigotas do parasita quando comparada aos ensaios *in vitro* do medicamento isolado. Este estudo demonstrou *in vitro* uma ação potencializadora do flavonoide nos ensaios *in vitro* de miltefosina, abrindo espaço para novas investigações sobre a associação de compostos naturais com drogas leishmanicidas.

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Palavras-chave: Naringenina; Miltefosina; Leishmaniose; Leishmaniose cutânea;

Leishmania amazonensis

ABSTRACT

Leishmaniasis is a disease caused by the unicellular protozoan of the genus Leishmania, with the cutaneous form being the most prevalent globally. One of the species, Leishmania amazonensis, is responsible for different clinical manifestations in Brazil such as localized cutaneous leishmaniasis (LCL), disseminated cutaneous leishmaniasis (LD), and diffuse cutaneous leishmaniasis (DCL). The first and second line drugs most commonly used in the treatment of leishmaniasis are highly toxic and show low efficacy in curing patients with the DCL form. Therefore, it is necessary to explore new therapies using less toxic leishmanicidal compounds, such as flavonoids, and their potential combinations with conventional drugs. In this study the main objectives were first to explore by literature review the *in vitro* action of flavonoids extracted from natural sources in Leishmania amazonensis and seek the main methodologies used for the study of these compounds. Another objective was to investigate the potential synergistic effect between miltefosine and the flavonoid naringenin. *In vitro* results indicated that naringenin has an inhibitory concentration (IC_{50}) of 219.9 μM and miltefosine has an IC_{50} of 13.21 μM. In the in silico analysis, Lipinski's rule of five was applied to assess the oral absorption of naringenin, which met all five criteria for this qualitative analysis. *In vitro* tests were conducted to construct an isobologram, resulting in the calculation of the dose reduction index (DRI) and fractional inhibitory concentration index (FICI), which demonstrated that the combination of naringenin with miltefosine produced an additive effect. This combination was able to reduce the IC_{50} of miltefosine by approximately two fold in the promastigote forms of the parasite, compared to the *in vitro* tests of the drug alone. This study demonstrated a potentiating effect of the flavonoid in combination with miltefosine *in vitro*, paving the way for future investigations on the association of natural compounds with leishmanicidal drugs.

Key words: Naringenin; Miltefosine; Leishmaniasis; Cutaneous leishmaniasis; Leishmania amazonensis

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1 INTRODUCTION

Leishmaniasis is a disease in which some species of the etiological agent present in Brazil and other countries are resistant to both first-and second-line drugs (WHO, 2023). Among first-line drugs, miltefosine has demonstrated cure rates similar to those of meglumine antimoniate. Its oral administration makes it a more attractive option. It has been shown to be effective against *Leishmania amazonensis* in both *in vitro* and *in vivo* tests on a parasite isolated from a patient in Brazil, successfully eliminating it (COELHO et al., 2014). However, miltefosine also presents a challenge due to the emergence of resistant strains resulting from its intensive and indiscriminate use (SUNDAR & MURRAY). A strategy already used in the treatment of bacterial and viral diseases, as well as breast cancer, is the use of combination therapies, which help mitigate side effects and reduce the emergence of resistant strains (JEAN et al., 2016; FISUSI & AKALA, 2019; SHYR et al., 2021). While drug combinations offer several advantages, there are also less toxic compounds from natural sources with leishmanicidal activity, such as naringenin (KAUR, CHAUHAN & KAUR, 2018). Given the challenges associated with other drugs, this study aims to explore the action of several types of flavonoids provided by plants in *L. amazonensis* are more investigate the effects of combining miltefosine with the flavonoid naringenin, with the goal of developing a new treatment approach and optimize the use of miltefosine in combating *L. amazonensis*.

2 LITERATURE REVIEW

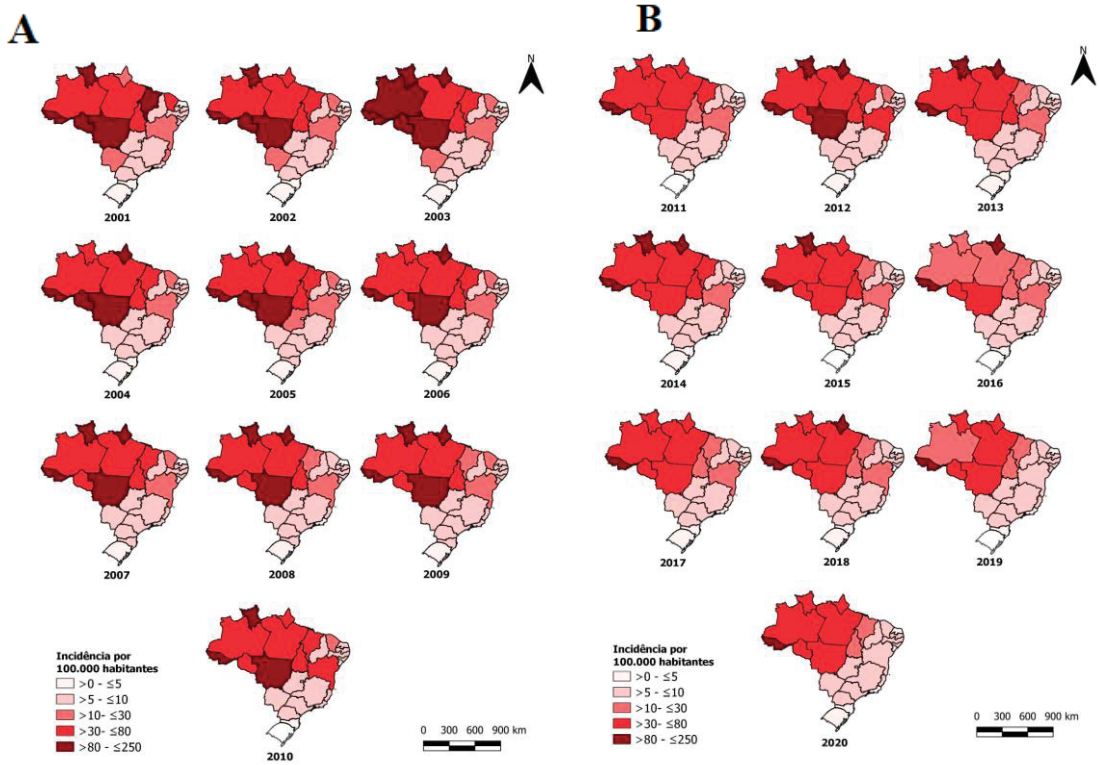
2.1 INCIDENCE OF VISCERAL AND CUTANEOUS LEISHMANIASIS

Leishmaniasis is a disease caused by a unicellular protozoan from the family *Trypanosomatidae*, belonging to the genus *Leishmania*. The three main forms of the disease are visceral leishmaniasis (VL), mucocutaneous leishmaniasis (ML), and cutaneous leishmaniasis (CL), leishmaniasis is classified as a neglected disease, with an annual record of 0.2 to 0.4 million cases of VL, occurring in ten countries: Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan, and Sudan. CL, the most common form, accounts for 0.7 to 1.2 million cases annually and is more widely distributed across four continents (WHO, 2019). The countries with the highest estimated burden of CL worldwide include Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Syria, North Sudan, and Peru (ALVAR et al., 2012; GRIFFERTY et al., 2021). Large portions of the world are at

potential risk for disease transmission, with several factors contributing to its spread, including poor sanitation, limited access to healthcare for low-income populations, and malnutrition (BOELAERT et al., 2009; GRIFFERTY et al., 2021; HERRERO et al., 2009; PIGOTT et al., 2014). Among the 100 million people in the Americas living on less than 1 US$ per day, 1% are affected by cutaneous leishmaniasis (HOTEZ et al., 2012; HOTEZ et al., 2013; STOLK et al., 2016).

In Brazil, a total of 431,885 cases of leishmaniasis were reported between 2001 and 2020 (Figure 1), with 878 recorded deaths, resulting in a lethality rate of 0.18%. The disease was reported in all five regions of the country. The northern region had the highest number of cases, with 182,398, and the highest incidence rate, at 11,149.73 cases per 100,000 inhabitants. However, it also had the lowest lethality rate, at 0.07%. In contrast, the states of Tocantins and Mato Grosso had the highest mortality rates from the disease in the country. Regarding lethality, the states of Sergipe, Rio Grande do Sul, and São Paulo, despite their relatively low incidence rates of 14.7/100,000, 1.60/100,000, and 20.59/100,000, respectively, exhibited the highest lethality rates compared to other states (BELO et al., 2023).

FIGURE 1: CHOROPLETHIC MAP SHOWING THE ANNUAL INCIDENCE RATE OF LC OF (X 100000 INHABITANTS) IN THE 27 UNITS

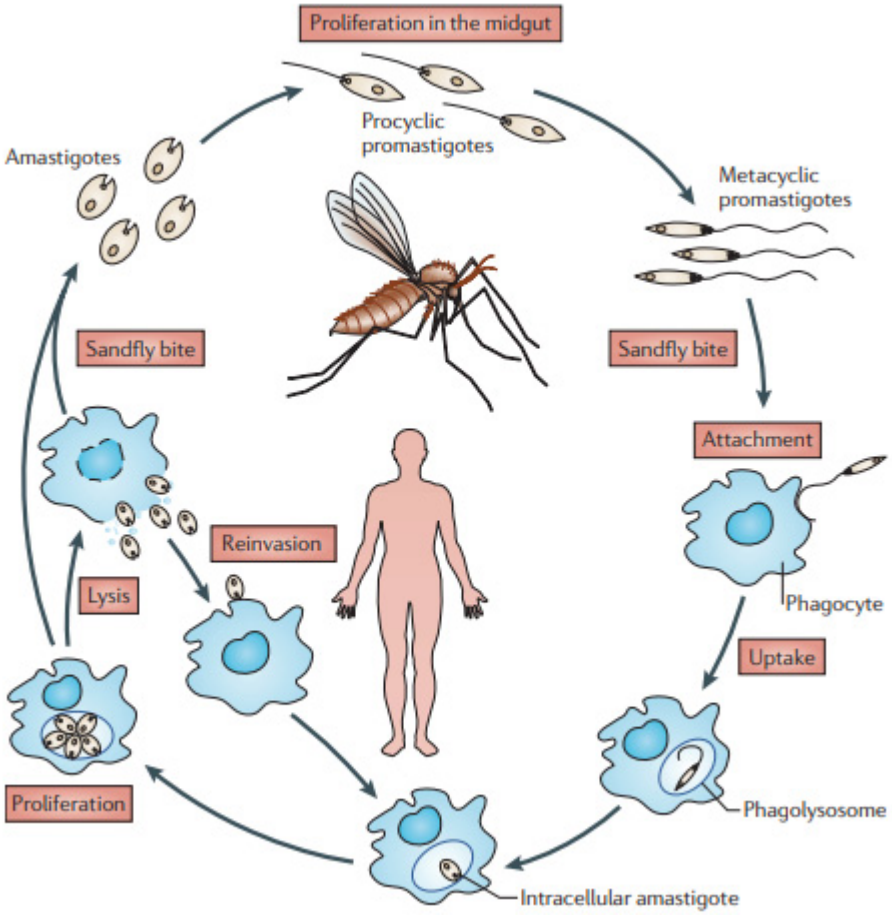


Source: (Adapted from Belo, et al., 2023)

2.2 LIFE CYCLE, SPECIES AND VECTORS OF CL IN BRAZIL

The life cycle of the parasite is divided into two stages (Figure 2): the insect vector, where the predominant forms are free-living, non-infectious procyclic promastigotes and the infective metacyclic promastigotes. In the mammalian host, where the parasite exists as intracellular amastigotes (LAISON, RYAN & SHAW, 1987). The cycle begins when a female sandfly takes a blood meal from an infected individual, ingesting macrophages that contain the amastigote forms. In the insect's midgut, these infected cells are lysed, releasing the amastigotes, which then differentiate into non-infectious reproductive promastigotes. These forms later transform into metacyclic promastigotes, which migrate from the intestine to the pharynx and eventually to the insect's oral cavity (ELNAIEM, WARD & YOUNG, 1992). When the infected female sandfly bites a new host, it inoculates the parasite along with its saliva. The insect’s saliva has chemoattractant properties, promoting the migration of phagocytes, such as neutrophils and macrophages, to the bite site a factor that facilitates parasite infection (ANJILI et al., 1995; PETERS et al., 2008). After being phagocytosed by innate immune cells, the metacyclic promastigotes differentiate into amastigotes. These forms evade elimination by reactive oxygen species (ROS) and nitric oxide (NO) within the parasitophorous vacuole of phagocytes due to the protective role of lipophosphoglycans (LPGs) on their surface (DESJARDINS & DESCOTEAUX, 1997).

FIGURE 2: LIFE CYCLE OF THE ETIOLOGICAL AGENT CAUSING LEISHMANIASIS.



Source: Adapted (Moreira; Batistela,2011)

In the Americas, 18 countries are endemic for cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (CML), with a total of 1,028,054 cases reported between 2001 and 2019. The countries with the highest numbers of cases are Brazil, Colombia, Peru, Nicaragua, and Bolivia, which together account for 77% of cases in the region (PAHO, 2020). In Brazil, the three main species responsible for CL are *Leishmania braziliensis*, *Leishmania guyanensis*, and *Leishmania amazonensis* (Brasil, 2017). Among these, *L. amazonensis* is prevalent in both primary and secondary forest areas of the Legal Amazon region (Amazonas, Pará, Rondônia, Tocantins, and Maranhão). It is also found in other regions, including the Northeast (Bahia), Southeast (Minas Gerais and São Paulo), Center-West (Goiás), and South (Paraná). This species is responsible for a broad spectrum of clinical forms, including localized cutaneous leishmaniasis (LCL), CML, diffuse cutaneous leishmaniasis (DCL), and disseminated leishmaniasis (DL).

The parasites are transmitted by female mosquitoes of the genus *Lutzomyia*, which serve as vectors for *Leishmania* species present in the New World. Leishmaniasis is primarily a zoonotic infection, mainly affecting wild animals and, secondarily, humans and domestic animals (BASANO & CAMARGO, 2004; LEWIS, 1971). In Brazil, *Lutzomyia flaviscutellata* is the main vector of *L. amazonensis* in the Legal Amazon. This phlebotomine sandfly feeds at ground level on a wide variety of animals, including marsupials and birds, and is particularly attracted to rodents (LAISON & SHAW, 1968). *Lutzomyia whitmani*, also considered an important vector for *L. braziliensis*, is widely distributed across Brazil, with a high frequency in animal shelters and a greater predominance in peridomestic areas (TEODORO et al., 2003). *Lutzomyia umbratilis*, the primary vector of *Leishmania. guyanensis*, is the main cause of human cutaneous leishmaniasis north of the Amazon River. However, compared to other vector species, its impact is more limited, as it is restricted to the northern region of the country (PINHEIRO, LUZ & RAMOS, 2008).

2.3 IMMUNOLOGICAL PROFILE AND SYMPTOMS OF LCD

The cure of leishmaniasis depends on the type of cellular immune response the patient develops, with both T helper 1 (Th1) and T helper 2 (Th2) responses being reported. A Th1-mediated response is associated with high levels of cytokines such as interleukin-12 (IL-12), interleukin-1 (IL-1), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) (VON S. E. et al., 2003). The predominance of a Th1 response is linked to disease resolution and clinical cure (TARAGHIAN, M. et al., 2021). IL-12 plays a crucial role in Th1 differentiation and proliferation, leading to IFN-γ production. IFN-γ stimulates the generation of superoxide (O_2^-) and nitric oxide (NO), which exert cytotoxic effects on the parasite. This process is further enhanced by TNF-α expression (MANSUETO et al., 2007). Patients with active lesions who did not achieve clinical cure exhibited lower levels of IL-12, IFN-γ, and TNF-α compared to those who were cured (TARAGHIAN, M. et al., 2021).

In contrast, a Th2-mediated response is characterized by the production of IL-4, which promotes the differentiation of T helper 0 (Th0) lymphocytes into Th2 cells, and IL-10, which exerts an anti-inflammatory effect by suppressing ROS, IL-12, and TNF-α production. IL-10 is highly expressed in patients who fail to achieve cure, contributing to the parasite's persistence (SACKS; NOBEN-TRAUTH, 2002; TARAGHIAN, M. et al., 2021). Diffuse cutaneous leishmaniasis (DCL) is a rare clinical form, characterized by widespread nodules and papules, predominantly affecting the extremities, with infrequent involvement of the

nasopharyngeal mucosa (SILVEIRA, 2009; SILVEIRA; LAINSON; CORBETT, 2004). Among the clinical manifestations caused by *Leishmania amazonensis*, DCL is particularly difficult to treat with conventional therapies. This form is characterized by a strong Th2 immune response, with high IL-10 and IL-4 expression and low IFN-γ levels, indicating patient anergy to the parasite. DCL also presents high parasite loads and disfiguring lesions, leading to significant physical and psychological impacts (BOMFIM et al., 1996; Brasil, 2017). In addition to the ineffective immune response in DCL, treatment with first-line drugs such as meglumine antimoniate and second-line therapies, including amphotericin B and its liposomal formulation, has shown limited efficacy in disease management (COSTA et al., 2009).

2.4 IMPACT OF LC ON THE PHYSICAL AND MENTAL HEALTH OF HUMAN PATIENTS

In Brazil, cutaneous leishmaniasis (CL) predominantly affects individuals with low educational attainment who rely on agricultural work as their main source of income. These individuals often belong to economically vulnerable population groups. The disease disproportionately affects non-white individuals, who account for approximately 65.81% of cases. Additionally, 52.64% of cases occur in rural areas, while 43.27% are reported in urban settings (OLIVEIRA et al., 2016; MELO et al., 2020; VASCONCELOS; ARAÚJO; ROCHA, 2017; BELO et al., 2023).

Although CL has a low mortality rate, it can cause severe skin deformities, including permanent marks and scars, leading to significant social stigma. The psychological impact on affected individuals is substantial, with documented cases of self-repulsion and, in severe instances, suicidal ideation (BENNIS et al., 2017). The social, physical, and psychological burdens imposed by CL have far-reaching consequences for both individual well-being and national productivity. Despite its low lethality, the disease's high morbidity affects individuals' overall health, often impairing their capacity to work. This, in turn, negatively impacts Brazil's economy (BEZERRA et al., 2018).

2.5 CHALLENGES IN THE TREATMENT OF LC

The World Health Organization (WHO) recommends pentavalent antimonials as the first-choice treatment for cutaneous leishmaniasis (CL), with two formulations: meglumine

antimoniate and stibogluconate, which are administered intramuscularly or intravenously (WHO, 2023). The recommended dosage is calculated in milligrams of pentavalent antimony (Sb+5) per kilogram of body weight per day (mg Sb+5/kg/day) (WHO, 1990). Other treatment options include miltefosine, amphotericin B, and liposomal amphotericin B (PAHO, 2020; SANTIAGO; PITA; GUIMARÃES, 2021). Due to the widespread use of Sb+5 in the treatment of all forms of leishmaniasis, a 65% failure rate for visceral leishmaniasis (VL) treatment with pentavalent antimoniate was reported in Bihar, India, and this failure is often attributed to the incorrect administration of the drug, such as initiating treatment with lower doses and increasing them over time or implementing drug-free intervals based on the belief that renal toxicity could be prevented (LIRA et al., 1999). In Brazil, resistance of *L. braziliensis* and *L. guyanensis* to meglumine antimoniate has been observed (ROMERO; GUERRA; MACÊDO, 2001). Besides meglumine antimoniate, pentamidine isethionate is also used as a first-choice drug for leishmaniasis, administered intravenously or intramuscularly. However, despite its effectiveness, relapse cases are common (Brasil, 2017). Treatment of cutaneous leishmaniasis caused by *L. amazonensis* with first and second-choice drugs presents significant challenges, including toxic effects and limited access to medical care for economically vulnerable populations, particularly in the north and northeast regions of Brazil. These regions face a shortage of resources and healthcare professionals (COSTA et al., 2009; OLIVEIRA et al., 2009).

While standard doses of meglumine antimoniate are generally effective, they are associated with adverse effects such as arthralgia, myalgia, and fever. More severe side effects, including acute pancreatitis, cardiomyopathy, and renal and hepatic failure, have also been reported (MACHADO et al., 2010; OLIVEIRA et al., 2009). Similarly, pentamidine isethionate can induce diabetes mellitus, nephrotoxicity, and thrombocytopenia, while amphotericin B is known for its nephrotoxic and cardiotoxic effects (HUGHES et al., 1978; MILDER; WALZER; POWELL, 1979; SAMPAIO et al., 1971).

Given these challenges, new therapeutic targets, such as miltefosine, have been explored. Miltefosine is orally administered, offering an advantage for individuals in remote areas with limited access to healthcare facilities and a shortage of healthcare professionals (CREMESP, 2020; FILHOS; LUCAS; SAMPAIO, 2008).

Miltefosine is approved by the U.S. Food and Drug Administration (FDA) for the treatment of cutaneous leishmaniasis and for veterinary use in Brazil, endorsed by the

Ministry of Health and the Ministry of Agriculture, Livestock and Supply (MAPA). It is favored for its low toxicity, ease of oral administration, and efficacy in eliminating the parasite, comparable to first and second-choice drugs (CRMV, 2016; FDA, 2014). *In vitro* and *in vivo* studies have demonstrated miltefosine's effectiveness in eliminating parasites isolated from patients with leishmaniasis (COELHO et al., 2014).

Studies comparing the cure rates of miltefosine and meglumine antimoniate show similar outcomes, for example, a cure rate of 63.15% (12/19) are reported in the miltefosine group, compared to 55.55% (5/9) for the meglumine antimoniate group (CHRUSCIAK-TALHARI et al, 2011). Similarly, other study found cure rates of 75% (45/60) for the miltefosine group, compared to 53.3% (16/30) for those treated with meglumine antimoniate (MACHADO et al.,

2010). The most common adverse effects of miltefosine include gastrointestinal symptoms such as vomiting, nausea, and abdominal pain, as well as teratogenic effects, which require caution in women of childbearing age. However, miltefosine’s adverse effects are generally milder than those associated with other treatments, making it a favorable option for treating clinical forms of *L. (L.) amazonensis* (MACHADO et al., 2010; OLIVEIRA et al., 2009; SINDERMANN; ENGEL, 2006).

Despite its advantages, the use of miltefosine in India as a first-line treatment led to a decrease in cure rates and an increase in relapses over a decade, due to the emergence of resistant strains of *Leishmania donovani*. Resistance has also been reported in *Leishmania infantum* isolates in Brazil (CARNIELLI et al., 2019).

To optimize leishmaniasis treatment, combination therapy has been explored. This approach improves effectiveness, reduces treatment costs and duration, and prevents the emergence of resistant strains. Combined treatments can enhance leukocyte activity and replication, increase cytokine production that regulates the Th1 response, and boost the production of reactive oxygen species (ROS) that help eliminate the parasite (VAN GRIENSVEN et al., 2010).

In addition to drug combinations, research has focused on finding antiparasitic compounds in plants, particularly flavonoids. These compounds, synthesized through the phenylpropanoid pathway, possess various pharmacological activities (LIU, W et al., 2021).

Flavonoids such as quercetin, cynaroside, and naringenin have shown leishmanicidal effects. Quercetin increases ROS production in *Leishmania (L.) amazonensis* and *Leishmania braziliensis* promastigotes, while interfering with iron metabolism in *L. braziliensis*. Cynaroside inhibits the enzyme udp-galactopyranose mutase (UGM) in *Leishmania donovani*, and naringenin promotes apoptosis-like effects, ROS production in macrophages, and an increase in cytokines involved in the Th1 response. Furthermore, these flavonoids have lower cytotoxicity compared to conventional leishmaniasis drugs (FONSECA et al., 2011; CATANEO et al., 2019, KAUR et al., 2018).

Given the efficacy of drug combinations and the leishmanicidal properties of flavonoids, this study aims to investigate the *in vitro* combinatory effect of naringenin and miltefosine in the treatment of *L. amazonensis*.

3 HYPOTHESIS

"The leishmanicidal effect of naringenin may optimize the action of miltefosine against *L. amazonensis in vitro*."

4 OBJECTIVES

4.1 GENERAL OBJECTIVES

- To evaluate the combinatory effect of naringenin with miltefosine in *in vitro* assays with *L. amazonensis.*

4.2 SPECIFIC OBJECTIVES

- To evaluate the therapeutic dose-response/treatments in promastigote forms with the flavonoid, naringenin and miltefosine alone.

- To evaluate the interaction between naringenin and miltefosine through a synergism assay

• To evaluate the dose reduction index (DRI) for the purpose of optimizing the dose of miltefosine when combined with naringenin.

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Esta seção apresenta os artigos desenvolvidos ao longo do doutorado e publicados. A formatação dos artigos segue as normas da revista que os artigos foram submetidos e/ou publicados.

6 CHAPTER I: FLAVONOIDS IN THE TREATMENT OF *LEISHMANIA AMAZONENSIS*: A REVIEW OF EFFICACY AND MECHANISMS

ABSTRACT

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania.* In recent years, natural compounds have attracted significant interest due to their potential efficacy and lower toxicity compared to synthetic chemical compounds. This review analyzed studies retrieved from the PubMed and Google Scholar databases, focusing on the use of flavonoids against *Leishmania amazonensis.* Studies that tested flavonoids with known susceptibility against the parasite were included and classified based on their ability to kill it. According to the criteria established for identifying the most comprehensive studies, 52 were included in the analysis. Of these, three studies met at least 13 of the evaluation parameters (70%) and were considered the most complete. Analysis of IC_{50} values reported in these articles revealed the activity of 69 flavonoids. Among the assays on amastigote forms, 33 reported high activity, and six reported moderate activity. For assays on promastigote forms, 32 experiments reported high activity, 16 showed moderate activity, and two demonstrated weak activity. Among the flavonoids tested, morelloflavone-4'"O-b-D-glycosyl and pinostrobin showed the highest activity, while naringenin exhibited the weakest activity, specifically against promastigote forms. Regarding cytotoxicity assays, the highest selectivity indices reported in the articles were for carajurin and luteolin. This review emphasizes the importance of studying flavonoids, particularly those extracted from plants and propolis, as a means to advance our understanding and treatment of *L. amazonensis* infections.

Keywords: : Natural compounds, Flavonoids, *Leishmania amazonensis*, *In vitro* assays.

6.1 INTRODUCTION

Leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, is a significant public health concern affecting millions of people worldwide. More than 1 billion individuals are at risk of contracting leishmaniasis due to living in areas where the disease is endemic (1). Every year, an estimated 30,000 new instances of visceral leishmaniasis (VL) and over one million new cases of cutaneous leishmaniasis (CL) are reported (1–3).

Leishmania species are typically divided into two primary groups: Old and New World species. The Old World species are found in Africa, Asia, the Mediterranean region, and the Middle East and include *Leishmania tropica*, *Leishmania major*, *Leishmania aethiopica*, and *Leishmania donovani* (4,5). The New World species, which are endemic to the Americas, include *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania panamensis*, *Leishmania peruviana*, *Leishmania guyanensis*, *Leishmania pifanoi*, *Leishmania venezuelensis*, *Leishmania shawi*, and *Leishmania lainsoni* (4,5).

Leishmaniasis is considered a neglected tropical disease, with most cases occurring among populations with low socioeconomic status. The disease manifests in three main clinical forms: VL, mucocutaneous leishmaniasis (MCL), and CL (6). Several factors contribute to the global spread of the disease, including limited access to healthcare among impoverished communities, poor nutrition, and inadequate sanitation (7–10). The vectors responsible for transmitting New World species are sandflies of the genus *Lutzmyia*. These parasites primarily infect animals, with humans becoming involved secondarily (11,12).

In Brazil, the disease disproportionately affects individuals with low education levels, economic vulnerability, and poor employment conditions, primarily in rural areas (13–15). The consequences of CL are both physical and psychological, impacting not only the health of patients but also the economy of the affected regions. CL presents high morbidity, which can interfere with the patient's physical condition and work productivity, leading to significant economic losses (16). Among the various species responsible for the disease, *L. amazonensis* is particularly noteworthy due to its high prevalence in the New World and its association with CL (17). This form manifests as chronic skin lesions, which can lead to severe disfigurement and social stigma, underscoring the urgent need for effective therapeutic interventions (2,5,18).

Leishmania amazonensis causes severe cutaneous lesions in mice and can induce the immune system to produce a mixed cytokine profile (19). The cytokines secreted in response to this species play a crucial role in the parasite's lifecycle, facilitating tissue invasion, nutrient

acquisition, and evasion of the host immune response. The anergic nature of *L. amazonensis* remains unclear, although several mechanisms have been proposed (20,21).

The cure of CL depends on the type of immune response, particularly one mediated by T helper 1 (Th1) cells (22). The Th1 response is characterized by high levels of cytokines such as interleukin-12 (IL-12), which promotes the differentiation of T Helper 0 (Th0) cells into Th1 cells; interleukin-1 (IL-1); and interferon-gamma (INF-γ), which stimulates the production of superoxide (O^{-2}) and nitric oxide (NO)—key components for parasite elimination by phagocytes. Tumor necrosis factor-alpha (TNF-α) further enhances the production of superoxides (23,24). In contrast, patients who do not achieve clinical cure typically exhibit a dominant T helper 2 (Th2)-mediated response, with elevated expression of interleukin-10 (IL-10), which promotes an anti-inflammatory effect that hinders effective parasite clearance (22,25).

This species has been identified in patients with diverse clinical forms of the disease, including localized cutaneous leishmaniasis (LCL), anergic diffuse cutaneous leishmaniasis (ADCL), MCL, and canine visceral leishmaniasis (CVL), particularly in South American countries, mainly Brazil (21,26). Among these, ADCL is the most difficult clinical form to treat with conventional drugs (22,25). It is characterized by numerous nodules and lesions covering large body areas (26,27). In ADCL patients, there is elevated expression of interleukin-4 (IL-4) and IL-10, along with low expression of IFN- γ, which reflects the anergic immune response typical of this condition (28). In fact, *Leishmania infantum* and *L. amazonensis* can cause the visceral form in dogs; in addition, *L. amazonensis* exhibits natural resistance to antileishmanial drugs, which may contribute to therapeutic failure (21,29).

There are only a few medications available to treat leishmaniasis, such as pentavalent antimony (SbV) compounds, which have remained the first-line treatment for several decades in some endemic areas, including Brazil, despite their low efficacy rates (29,30). In addition to the ineffective immune response to ADCL caused by *L. amazonensis*, first-line drugs like meglumine antimoniate, as well as second-line treatments such as amphotericin B and liposomal amphotericin, are ineffective for treating this clinical form (31).

Current treatment options for leishmaniasis predominantly rely on chemotherapeutic agents, including SbV compounds, amphotericin B, and miltefosine (32). However, serious side effects are associated with many standard formulations, including meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®), as well as alternative medications like liposomal amphotericin B (AmBisome®), pentamidine, allopurinol, paromomycin, and azole derivatives (33,34). These treatments face several challenges,

including high toxicity, variable efficacy, and the development of drug resistance (35,36). The invasive nature of these treatments and their significant side effects also complicate patient compliance and overall treatment success. Given these limitations, exploring alternative therapeutic strategies is imperative.

Natural compounds have attracted considerable interest in recent years due to their potential efficacy and lower toxicity profiles (37,38). Much of the knowledge regarding the therapeutic use of plants is transmitted orally through folklore, particularly in the Brazilian Amazon Forest. Plants represent a valuable resource for pharmacological research against parasites, given the long-standing coexistence of herbal treatments, humans, and parasitic diseases (37,38). Moreover, natural products provide exceptional structural diversity compared to conventional combinatorial chemistry, enabling the discovery of novel low molecular-weight lead compounds (38,39). It is estimated that nearly 90% of all plant species have yet to be explored for their potential as antileishmanial agents (40). Key factors driving the search for new drugs include limited access to chemotherapy for parasitic infections, the high cost of treatment adherence in endemic regions, increased travel to endemic areas, and the associated need for effective prophylaxis, as well as the growing resistance to conventional drugs (41,42).

Numerous plant-derived compounds and secondary metabolites, including terpenoids, flavonoids, alkaloids, and essential oils, have shown antileishmanial activity in both *in vitro* and *in vivo* studies (38,41,43,44). For example, berberine, a plant-derived alkaloid, exhibits significant leishmanicidal effects by inhibiting parasite growth and inducing apoptosis (4,38). Curcumin, a compound present in turmeric, possesses strong immunomodulatory and anti-inflammatory properties that increase its antileishmanial efficacy (4,37,45). Additionally, essential oils from *Artemisia annua* and *Melaleuca alternifolia* have shown notable antileishmanial activity (4,37,38).

Flavonoids are a class of natural polyphenolic compounds and secondary metabolites produced via the phenylpropanoid pathway in a wide range of plant species (46,47). They are classified into six major categories: (i) flavanones, (ii) flavones, (iii) isoflavones, (iv) flavonols, (v) flavanols, and (VI) anthocyanins (48). This group of natural compounds is of significant research interest due to its diverse biological activities and therapeutic potential. For example, quercetin, a flavonol found in many fruits and vegetables, has been shown to inhibit parasite proliferation, stimulate the production of reactive oxygen species (ROS) that induce cell death in *L. amazonensis*, and modulate host immune responses (41,43,44).

Naringenin, a citrus flavanone found abundantly in citrus fruits, is a glycosylated flavonoid formed by the flavanone naringenin and the disaccharide neohesperidoside. It is primarily derived from yellowish dihydroflavonoids extracted from the dried peel of Rutaceae plants and grapefruits (49–51). Naringenin has potent anti-inflammatory properties, making it effective in relieving and treating a wide range of inflammatory conditions, including airway inflammation (51). It also has neuroprotective and renal effects, as well as therapeutic potential in the prevention and management of metabolic syndrome and cardiovascular diseases (49,51). Its antioxidant and anti-inflammatory properties suggest potential applications in treating protozoan infections. Although further studies are needed to assess its efficacy against pathogens, naringenin may serve as a complementary agent alongside conventional treatments for leishmaniasis.

Despite growing interest in natural compounds for treating leishmaniasis, comprehensive evaluations of their efficacy, mechanisms of action, and potential as viable therapeutic agents remain limited. To address this gap, we conducted a systematic review of the literature focusing on the use of flavonoids against *L. amazonensis*. By advancing our understanding of the antileishmanial potential of these natural compounds, we hope to contribute to the development of safer, more effective, and more accessible treatment options for leishmaniasis.

6.2 METHODS

6.2.1 Identification and Selection of Studies

A systematic search was conducted in the MEDLINE (via PubMed) and Google Scholar databases to identify relevant studies on natural compounds used in the treatment of *L. amazonensis*. As this is a systematic review, ethical approval and informed consent were not required. All articles that matched the predefined keywords aligned with the study objective were considered for inclusion. This review adhered to the methodological guidelines outlined in the PRISMA Statement (52).

The search encompassed studies published up to June 2024 and focused on natural compounds with potential therapeutic effects against *L. amazonensis*. The search strategy, including indexed terms and the criteria for inclusion and exclusion, is detailed in Table 1. Additionally, references cited in the selected publications were screened for further relevant studies.

TABLE 1: SEARCH STRATEGIES AND INCLUSION AND EXCLUSION CRITERIA APPLIED IN THE SYSTEMATIC REVIEW UPON NATURAL COMPOUNDS USED IN LEISHMANIASIS (*L. AMAZONENSIS*) TREATMENT

Index Terms	
Pubmed	**Google Scholar**
(*Leishmania amazonensis*) AND (((biological products) OR (medicinal plant) OR (natural compounds))) OR (Chemical treatment) OR (Flavonoid) OR (Cutaneous diffuse) OR (Synergism)	(*Leishmania amazonensis*) (biological products or medicinal plants or natural compounds)
Applied Criteria	
Inclusion	**Exclusion**
Studies evaluating natural compounds for antileishmanial activity. Studies assessing the synergistic effects of natural compounds combined with commercial drugs.	Studies involving other *Leishmania* species. Studies testing synthetic chemical compounds. Dissertations, theses, review articles, book chapters, and letters to the editor.

Source: The autor

6.2.2 Methodological quality assessment

The methodological quality of the studies included in this review was assessed independently by two reviewers (Vinícius Lessa and Guilherme Drescher – VL and GD, respectively). The evaluation focused specifically on studies that tested flavonoids against *L. amazonensis*, with inclusion limited to those using *in vitro* assays.

For each article, we examined the type of solvent used for the extraction and isolation of the compounds, as well as the methods used to characterize the flavonoids. We also identified the type of diluents employed to dissolve the flavonoids for testing purposes. Particular attention was paid to whether the studies used colorimetric assays to evaluate antileishmanial activity and assessed the cytotoxic concentration 50 (CC_{50}) in mammal cells.

Furthermore, we verified whether the studies reported the inhibitory concentration 50 (IC_{50}) against promastigote and amastigote forms and whether CC_{50} values were also determined. In all selected articles, we investigated whether the selectivity index (SI) was calculated for either isolated flavonoids or mixtures present on the solvent extracts from biological material.

Additionally, we evaluated if the studies conducted synergism assays in promastigote and amastigote forms to determine the interaction type between flavonoids, as well as between flavonoids and commercial drugs. We also extracted information regarding any

proposed mechanism of actions against both parasite forms, and if any *in silico* assays were performed. Finally, we checked which types of experimental controls were used in each study.

If required, additional information was requested from the authors of the included studies. Any discrepancies in data extraction were resolved through group discussion, with the assistance of a third evaluator.

6.2.3 Activity against parasite and Cytotoxicity assays

For all studies in which flavonoids were characterized, we evaluated their antileishmanial activity based on their ability to inhibit parasite growth. An extract or compound was considered active if it exhibited an IC_{50} value of ≤10 µg/mL against promastigote or amastigote forms. Moderate activity was defined as an IC_{50} value between >10 µg/mL and <50 µg/mL, while weak activity was assigned to those with IC_{50} values between ≥50 µg /mL and 100 µg/mL. Only the IC_{50} values of the characterized flavonoids were included in this review.

To assess treatment efficacy, we considered the SI, where SI ≥ 10 indicates high therapeutic potential, as values above this threshold suggest that the compound is more selective toward the parasite than to host cells (4).

To determine the nature of the interaction between natural compounds, we adopted the fractional inhibitory concentration index (FICI). A ΣFICI ≥ 0.5 indicates a synergistic effect, 0.5 > ΣFICI < 4 indicates an additive effect, and ΣFICI > 4 denotes an antagonistic effect (53).

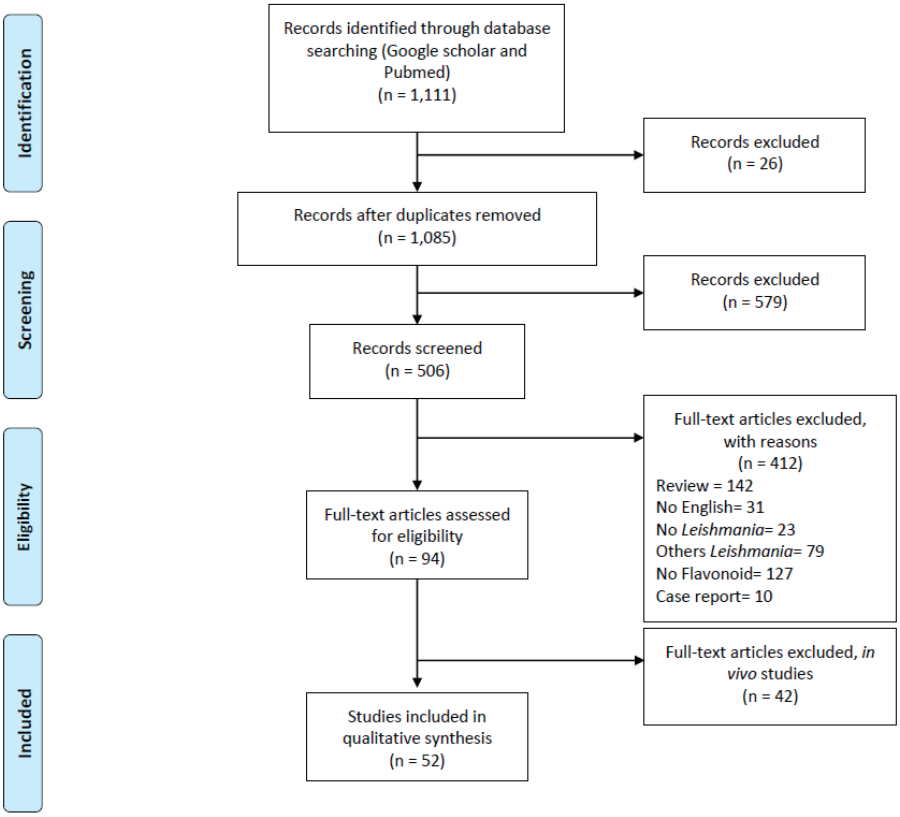
6.3 RESULTS

The initial search retrieved 208 articles from PubMed and 1,137 from Google Scholar databases. Of these, 579 titles or abstracts were initially retained for evaluation based on the search strategy. After removing 26 duplicates, 506 records remained for screening.

Out of the 506 records, 142 were excluded for being review articles, and 31 were excluded due to being published in languages other than English. An additional 23 studies were excluded for not addressing *Leishmania*, and 79 were excluded for working with *Leishmania* species other than *L. amazonensis*. A further 83 articles were excluded for not involving flavonoid compounds, and 10 papers were removed for being case reports. Ninety-

four full-text articles were assessed for eligibility, of which 42 were excluded for focusing on *in vivo* studies. Ultimately, 52 studies met the inclusion criteria and were included in the qualitative synthesis (Figure 1).

FIGURE 3: PRISMA FLOW DIAGRAM



Source: (based in Moher et al., 2009)

6.3.1 Quality assessment of included studies

Three articles met at least 13 of the 18 quality assessment criteria (70%) listed in Supplementary Tables S1 and S2 and were classified as the most complete studies. Twenty-six articles met between nine and 12 criteria (50–65%) and were categorized as regular studies. The remaining 23 articles met eight or fewer criteria (45–23%), suggesting a lower level of methodological completeness (see Supplementary Table S1).

Among the 52 included studies, 38 (73%) evaluated the antileishmanial activity of specific plant-derived fractions. Additionally, 41 papers (78%) specifically investigated the effects of isolated and characterized flavonoids against *L. amazonensis*.

TABLE 2 - LIST OF PAPERS WITH EACH ASSESSMENT OF QUALITY RESULTS BY SELECTED CRITERIA

	Solvent	Characterization Methods	Characterized Flavonoid	Diluent	Flavonoid Quantification	Colorimetric Methods for Citoxicity and Antileishmanial Assay	IC_{50} Amastigote	IC_{50} Promastigote	CC_{50}
Araújo et al 2024	Yes			Yes	Yes	Yes	Yes	Yes	Yes
Dutra et al 2023	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes
Pacheco et al 2023	Yes	Yes	Yes			Yes	Yes	Yes	Yes
Fróes et al 2023	Yes	Yes	Yes			Yes		Yes	Yes
Araújo et al 2022			Yes	Yes		Yes		Yes	Yes
Silva et al 2022	Yes	Yes	Yes			Yes		Yes	Yes
Bezerra et al 2021	Yes	Yes	Yes	Yes		Yes		Yes	Yes
Rizk et al 2022	Yes		Yes	Yes		Yes		Yes	
Silva et al 2021	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes
Silva et al 2021	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes
Cavalcante et al 2021	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Rizk et al 2021			Yes	Yes		Yes	Yes		Yes
Ferreira et al 2021	Yes	Yes	Yes			Yes		Yes	Yes
Morais et al 2020	Yes	Yes	Yes			Yes	Yes	Yes	Yes
Silva et al 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes
Santos et al 2019	Yes	Yes		Yes		Yes	Yes	Yes	Yes
Rocha et al 2019		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Emiliano & Almeida-Amaral 2018			Yes	Yes	Yes	Yes	Yes		
Almeida-Souza et al 2018	Yes	Yes		Yes		Yes	Yes		
Fadel et al 2018	Yes			Yes	Yes	Yes	Yes		Yes
Delgado-Altamirano	Yes	Yes		Yes		Yes	Yes	Yes	Yes

et al 2017									
Cuesta-Rubio et al 2017	Yes	Yes		Yes		Yes	Yes	Yes	Yes
Correia et al 2016	Yes			Yes	Yes	Yes		Yes	Yes
Duarte et al 2016	Yes	Yes			Yes	Yes	Yes	Yes	Yes
Fonseca-Silva et al 2016			Yes	Yes	Yes		Yes		
Fonseca-Silva et al 2015			Yes	Yes	Yes			Yes	
Mai et al 2015	Yes	Yes	Yes	Yes	Yes	Yes		Yes	
Rizk et al 2014	Yes	Yes	Yes	Yes			Yes		
Assolini et al 2020			Yes	Yes		Yes	Yes	Yes	Yes
Zeouk et al 2020	Yes	Yes	Yes	Yes			Yes	Yes	Yes
Oliveira et al 2021	Yes	Yes	Yes				Yes	Yes	
Fadel et al 2019	Yes			Yes	Yes		Yes	Yes	Yes
Araújo et al 2019	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Cabanillas et al 2014	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Dal Picolo et al 2014	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Ribeiro et al 2014	Yes	Yes	Yes	Yes		Yes		Yes	Yes
Wong el al 2014		Yes		Yes		Yes	Yes	Yes	Yes
Lage et al 2013	Yes	Yes	Yes	Yes		Yes		Yes	Yes
Manjolin et al 2013		Yes	Yes	Yes			Yes		
Gervazoni et al 2018			Yes	Yes			Yes	Yes	Yes
Fabri et al 2009	Yes	Yes		Yes	Yes	Yes		Yes	Yes
Silva et al 2011			Yes	Yes		Yes		Yes	
Gontijo et al 2012	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Grecco et al 2012	Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Machado et al 2007	Yes	Yes	Yes	Yes				Yes	

Pereira et al 2011	Yes	Yes	Yes	Yes	Yes		Yes		
Salvador et al 2009	Yes	Yes	Yes	Yes		Yes	Yes		
Taled-Contini et al 2004	Yes	Yes	Yes	Yes	Yes	Yes		Yes	
Lessa et al 2024			Yes	Yes	Yes	Yes		Yes	
Inacio et al 2013			Yes	Yes	Yes		Yes		
Salvador et al 2002	Yes	Yes	Yes	Yes	Yes	Yes			
Clavin et al 2007	Yes	Yes	Yes	Yes					

Source: The autor

TABLE 3 - LIST WITH MORE SELECTED CRITERIA

	Selectivity Index (SI) Amastigote	**Selectivity Index (SI) Promastigote**	**Fractional Inhibitory Concentration Index (FICI) Amastigote**	**Fractional Inhibitory Concentration Index (FICI) Promastigote**	**Mechanism of Action in Promastigotes**	**Mechanism of Action in Amastigotes**	**In silico Assay**	**Control for Antipromastigotes Assay**	**Control for Antiamastigote Assay**
Araújo et al 2024	Yes	Yes				Yes		Yes	Yes
Dutra et al 2023	Yes						Yes	Yes	Yes
Pacheco et al 2023	Yes							Yes	Yes
Fróes et al 2023		Yes		Yes	Yes		Yes	Yes	
Araújo et al 2022		Yes			Yes			Yes	
Silva et al 2022		Yes			Yes		Yes	Yes	
Bezerra et al 2021	Yes					Yes			Yes
Rizk et al 2022					Yes	Yes	Yes	Yes	Yes
Silva et al 2021					Yes	Yes		Yes	Yes
Silva et al 2021	Yes					Yes	Yes	Yes	Yes
Cavalcante et al 2021								Yes	
Rizk et al 2021	Yes					Yes	Yes		Yes
Ferreira et al 2021		Yes						Yes	
Morais et al 2020	Yes				Yes			Yes	

Silva et al 2019	Yes						Yes	Yes	Yes
Santos et al 2019	Yes	Yes						Yes	
Rocha et al 2019						Yes		Yes	Yes
Emiliano & Almeida-Amaral 2018			Yes						Yes
Almeida-Souza et al 2018	Yes					Yes			Yes
Fadel et al 2018	Yes								
Delgado-Altamirano et al 2017	Yes	Yes						Yes	Yes
Cuesta-Rubio et al 2017		Yes						Yes	Yes
Correia et al 2016		Yes						Yes	
Duarte et al 2016						Yes		Yes	Yes
Fonseca-Silva et al 2016						Yes			Yes
Fonseca-Silva et al 2015					Yes			Yes	
Mai et al 2015								Yes	
Rizk et al 2014	Yes					Yes		Yes	Yes
Assolini et al 2020	Yes	Yes			Yes	Yes		Yes	Yes
Zeouk et al 2020	Yes	Yes			Yes				
Oliveira et al 2021								Yes	Yes
Fadel et al 2019								Yes	Yes
Araújo et al 2019		Yes			Yes	Yes		Yes	Yes
Cabanillas et al 2014	Yes	Yes	Yes			Yes			Yes
Dal Picolo et al 2014		Yes						Yes	Yes
Ribeiro et al 2014						Yes	Yes	Yes	Yes
Wong el al 2014									Yes

Lage et al 2013		Yes					Yes	Yes
Manjolin et al 2013						Yes		
Gervazoni et al, 2018	Yes					Yes	Yes	Yes
Fabri et al 2009							Yes	
Silva et al 2011				Yes			Yes	
Gontijo et al 2012					Yes		Yes	Yes
Grecco et al 2012								Yes
Machado et al 2007							Yes	
Pereira et al 2011							Yes	Yes
Salvador et al 2009							Yes	
Taled-Contini et al 2004							Yes	
Lessa et al 2024			Yes				Yes	
Inacio et al 2013						Yes		Yes
Salvador et al 2002								Yes
Clavin et al 2007								

Source: The autors

6.3.2 Activity against parasite and Cytotoxicity assays

Research articles were considered for inclusion if they demonstrated a significant antileishmanial effect and provided flavonoid characterization, including data from colorimetric and plate reader assays. These studies reported IC_{50} and CC_{50} values in *in vitro* experiments, as summarized in Supplementary Tables S3 and S4.

A total of 69 flavonoids were identified across the reviewed studies and evaluated for IC_{50} values against both amastigote and promastigote forms of *L. amazonensis*, as well as CC_{50} values in cytotoxicity assays, according to the criteria described in Section 2.3.

Among the assays targeting amastigotes, 33 showed high activity ($IC_{50} \leq 10$ μg/mL), six showed moderate activity (10 μg/mL < IC_{50} < 50 μg/mL), and none showed weak activity.

For the promastigote forms, 32 assays reported high activity, 16 showed moderate activity, and two were classified as weakly active.

The most active flavonoid against promastigotes was morelloflavone-4'"O-b-D-glycosyl, with an IC_{50} of 0.0285 µg/mL. For amastigotes, pinostrobin demonstrated the highest activity, with an IC_{50} of 0.0838µg/mL. Conversely, naringenin was the least active compound, with an IC_{50} of 59.87 µg/mL reported only for promastigotes.

Regarding cytotoxicity, the highest SI reported was 34.8 for carajurin against amastigotes and 32.4 against promastigotes. The lowest SI values were 1.1 for amastigotes and 0.41 for promastigotes, both associated with luteolin.

TABLE 4 - CHARACTERIZATION OF FLAVONOIDS WITH IC_{50} AND THE ACTIVITY LEVEL OF THE FLAVONOIDS FOR *L. AMAZONENSIS* AMASTIGOTE AND PROMASTIGOTE ASSAYS. * ND: NOT DEMONSTRED

	Characterized flavonoid	IC_{50} amastigote	IC_{50} promastigote	Activity of isolated flavonoids amastigote assay	Activity of isolated flavonoids promastigote assay
Dutra et al 2023	7,8,3′-trihydroxy-4′-methoxyisoflavone	ND	ND	ND	ND
	Calycosin	ND	16.11 ± 0.9 µM (4.58 ± 0.26 µg/mL)	ND	High
	Formononetin	ND	112.0 ± 7.8 µM (30.04 ± 2.09 µg/mL.)	ND	Moderate
	Biochanin	ND	ND	ND	ND
	Atalantoflavone (Erythrina sigmoidea)	3.6 ± 0.5 µM (1.211±0.168µg/mL)	ND	High	ND
Araújo et al 2022	(−)-duartin	ND	2.47 ± 0.92 µg/mL	ND	High
	(3R)-claussequinone	ND	37.15 ± 2.43 µg/mL	ND	Moderate
Silva et al 2022	Carajurin	ND	7.96 ± 1.23 µg/mL	ND	High
Rizk et al 2022	amentoflavone	ND	15.6 ± 1.1 µg/mL	ND	Moderate
Silva et al 2021	luteolin	11.78 ± 1.24 µg/mL	31.61± 1.13 µg/mL	Moderate	Moderate
	apigenin	ND	45.6 ± 1.08 µg/mL	ND	Moderate
Silva et al 2021	carajurin	7.065 ± 1.19 (µg/mL)	3.662 ± 1.16 (µg/mL)	High	High
Rizk et al 2021	Amentoflavone	2.3 ± 0.93 µM (1.24±0.50µg/mL)	ND	High	ND
Morais et al 2020	hemileiocarpin	1.13 (0.9 ±1.4) µg/mL (compound 3)	4.5 ± 0.5 µg/mL (compound 3)	High	High
	herein	ND	ND	ND	ND
	connarin	ND	ND	ND	ND
Silva et al 2019	Abyssinone IV (Erythrina sigmoidea)	14.7 ± 1.2 µM (4.940 ± 0.399µg/mL)	ND	High	ND
	Atalantoflavone (Erythrina sigmoidea)	3.6 ± 0.5 µM (1.211±0.168µg/mL)	ND	High	ND
	Eriodictyol (Vernonanthura tweedieana)	ND	ND	ND	ND
Rocha et al 2019	Brachydin A (dimeric flavonoid)	>20 µM (>10.45 µg/mL)	>20 (>10.45 µg/mL)	Moderate	Moderate
	Brachydin B (dimeric flavonoid)	2.20 ± 0.09 (1.18±0.05µg/mL)	9.16 ± 1 (4.91±0.54µg/mL)	High	High

	Brachydin C (dimeric flavonoid)	6.25 ± 1.28 (3.17±0.65µg/mL)	10 ± 0.8 (5.07±0.41µg/mL)	High	High
Emiliano_&_Almeida-Amaral 2018	Apigenin	3.85 µM (1.04 µg/mL.)	ND	High	ND
Fonseca-Silva et al 2016	Apigenin	4.3 *µ*g/mL	ND	High	ND
Fonseca-Silva et al 2015	Apigenin	ND	23.7 *µ*g/mL	ND	Moderate
Mai et al 2015	*G. oudiepe 1) 5,7-dihydroxy-3,3,4,6-tetramethoxyflavone*	ND	8.15 µM (3.05 *µ*g/mL)	ND	High
	2) 30,5,7-trihydroxy-3,4,5,6-tetramethoxyflavone	ND	10.23 µM (3.99 *µ*g/mL)	ND	High
	G. urvillei 3) 5,7-dihydroxy-3,3,4,5,6-pentamethoxyflavone	ND	9.65 µM (3.9*µ*g/mL)	ND	High
	4) 5,7-dihydroxy-3,3,4,5,6-pentamethoxyflavone	ND	21.77 µM (9.1 3.9*µ*g/mL)	ND	High
	5) 5,7-dihydroxy-3,3,4,5,6-pentamethoxyflavone	ND	31.61 µM (13.66 µg/mL	ND	Moderate
	6) 40,5,7-trihydroxy-3,6,8-trimethoxyflavone	ND	27.33 µM (9.84 µg/mL)	ND	High
	7) 40,5,7-trihydroxy-3,6-dimethoxyflavone	ND	8.07 µM (2.67 µg/mL)	ND	High
	8) 5,7-dihydroxy-3,4,6-trimethoxyflavone	ND	63.15 µM (21.73 µg/mL)	ND	Moderate
	9) 5,7-dihydroxy-3,4,6-trimethoxyflavone	ND	14.80 µM (5.3 µg/mL)	ND	High
	10) 5,7-dihydroxy-3,4,6-trimethoxyflavone	ND	37.17 µM (13.83 µg/mL)	ND	Moderate
	11) Comercial kaempferol	ND	27.56 µM (8.27 µg/mL)	ND	High
	12) 3-methoxy-kaempferol (kaempferol-3-monomethylether)	ND	14.29 µM (4.49 µg/mL)	ND	HIgh
	13) 3-methoxy-kaempferol (kaempferol-3-monomethylether)	ND	22.95 µM (7.53µg/mL)	ND	High
	14) 3-methoxy-kaempferol (kaempferol-3-monomethylether)	ND	33.00 µM (9,45µg/mL)	ND	High
	15) Semi-synthesis (triacetyl derivative)	ND	12.27 µM (5.57 µg/mL)	ND	High
	16) Semi-synthesis (tetraacetyl derivative)	ND	48.52 µM (14.56 µg/mL)	ND	Moderate
Rizk et al 2014	*Selaginella sellowii* hydroethanolic extract (SSHE)	20.2 µg/mL (µM)	ND	Moderate	ND
	Amentoflavone	0.1 ± 0.2 *µ*g/mL	ND	High	ND
	Robustaflavone	2.8 ± 5.3 *µ*g/mL	ND	High	ND
Assolini et al 2020	4-nitrochalcone (4NC) (comercial)	4.04 µM (1.02 µg/mL)	21.2 µM (5.37µg/mL)	High	High
	Kaempferol 7-*O*-methyl ether	ND	>100 µM (>30.03 µg/mL)	ND	Moderate
	Kaempferol 3,7- di-*O*-methyl ether	10.5±2.5 (3.30±0.79µg/mL)	54.2± 2.2 µM (17.04±0.69µg/mL)	High	Moderate
	Myricetin 3,7,3',4'-tetra-*O*	ND	>100 µM (>36.03 µg/mL)	ND	Moderate
	Gossypetin 3,7,8,4'-penta-O-methyl ether	ND	>100 µM (>33.23 µg/mL)	ND	Moderate
Dal Picolo et al 2014	Aduncha lcone	ND	97 ± 0.2 µg/µL (11.03 ± 2.11 µM)	ND	Moderate
	Brachydin B	2.20 ± 0.09 µM (1.18±0.05µg/mL)	9.16 ± 1 µM (4.91±0.54µg/mL)	High	High
	Brachydin C	6.25 ± 1.28 µM (3.17±0.65µg/mL)	10 ± 0.80 µM (5.07±0.41µg/mL)	High	High
Lage et al 2013	quercetin 3-O-methyl ether	ND	8.1 ± 1.5 µM (2.56±0.47µg/mL)	ND	High
	strychnobiflavone	ND	3.2 ± 0.2 µM (2.02±0.13µg/mL)	ND	HIgh

Manjolin et al 2013	Isoquercitrin	3.8 µM (1,76µg/mL)	ND	High	ND
	Quercitrin	10 µM (4,48µg/mL)	ND	High	ND
	7,8-dihydroxyflavone	12 ± 1 µM (3,05 ± 0,25 µg/mL)	ND	High	ND
	Orientin	16 ± 2 µM (7,17 ± 0,90 µg/mL)	ND	High	ND
	Isoorientin	9 ± 1 µM (4.04±0.45µg/mL)	ND	High	ND
	Fisetin	1.3 ± 0.3 µM (0,37 ± 0,09 µg/mL)	ND	High	ND
	Quercetin	4.3 µM (1,30 µg/mL)	ND	High	ND
	Luteolin	9 ± 1 µM (2,58 ± 0,29 µg/mL)	ND	High	ND
	Kaempferol	~50 µM (14,31 µg/mL)	ND	Moderate	ND
	Galangin	~100 µM (~27,02 µg/mL)	ND	Moderate	ND
Gervazoni; Ozório and Amaral, 2018	2'-Hydroxyflavanone	3.09 µM (0,74 µg/mL)	20.96 µM (5,04µg/mL)	High	High
Silva et al 2011	Quercetin	Not determined	31.4 µM (9,49 µg/mL)	High	High
Gontijo et al 2012	1) morelloflavone-4'"O-b-D-glycosyl	Not determined	0.0513 µM (0.0285 µg/mL)	high	High
	2) (±)-fukugiside	Not determined	0.0446 µM (0.0320 µg/mL)	High	High
	3) morelloflavone	0.29 µM (0.161µg/mL)	0.139 µM (0.0774µg/mL)	High	High
	4) Morelloflavone-7,4,7,3,4-penta-O-acetyl	0.042 µM (0.0234µg/mL)	0.0147 µM (0.00818µg/mL)	High	High
	5) Morelloflavone-7,4',7'",3'",4'"-penta-O-methyl	0.0603 µM (0.0335µg/mL)	0.0403 µM (0.0224µg/mL)	High	High
	6) Morelloflavone-7,4',7'",3'",4'"-penta-O-butanoyl	0.059 µM (0.0328µg/mL)	0.0189 µM (0.0105µg/mL)	High	High
Grecco et al 2012	Naringenin	ND	ND	ND	ND
	sakuranetin	51.89 (39.31–69.98) µg/mL	52.60* (37.82–75.20) µg/mL	Moderate	Low
Salvador et al 2009	1) Pinostrobin	0.31 µM (0.0838µg/mL)	ND	High	ND
	2) Pinocembrin	3.45 µM (0.884µg/mL)	ND	High	ND
	3) Tectochrysin	0.56 µM (0.150µg/mL)	ND	High	ND
	4) Galangin 3-methyl ether	2.89 µM (0.822 µg/mL)	ND	High	ND
Lessa et al 2024	Naringenin	Not determined	219.86 µM (59,87 µg/mL)	ND	Low

Source: The autor

TABLE 5: CHARACTERIZATION OF FLAVONOIDS WITH IC_{50} AND THE ACTIVITY LEVEL OF THE FLAVONOIDS FOR *L. AMAZONENSIS* AMASTIGOTE AND PROMASTIGOTE ASSAYS. * ND: NOT DEMONSTRED

	Characterized flavonoid	CC_{50}	SI (seletivity index) amastigote	SI (seletivity index) promastigote
Dutra et al 2023	7,8,3′-trihydroxy-4′-methoxyisoflavone	ND	ND	ND
	Calycosin	ND	ND	ND
	Formononetin	ND	ND	ND

	Biochanin	ND	ND	ND
	Atalantoflavone (Erythrina sigmoidea)	44.1 ± 4.9 (μM) (14.82±1.65μg/mL)	12.12	ND
Araújo et al 2022	(−)-duartin	346.41 ± 40.99 μg/mL	ND	9.3
	(3R)-claussequinone	387.79 ± 25.93 μg/mL	ND	157
Silva et al 2022	Carajurin	258.2 ± 1.20 μg/mL	ND	32.4
Rizk et al 2022	amentoflavone	ND	ND	ND
Silva et al 2021	luteolin	8.005 ± 1.23 μg/mL	0.679	ND
	apigenin	11.87 ± 1.32 μg/mL	ND	ND
Silva et al 2021	carajurin	16.48 ± 1.10 (μg/mL)	34.8	ND
Rizk et al 2021	Amentoflavone	22.3 μM (12.00 μg/mL)	greater than 10	ND
Morais et al 2020	hemileiocarpin	7.2 (5.4-9.6) (compound 3)	6.3	ND
	herein	ND	ND	ND
	connarin	ND	ND	ND
Silva et al 2019	Abyssinone IV (Erythrina sigmoidea)	79.8 ± 2.4 (μM) (31.32±0.94μg/mL)	5.43	ND
	Atalantoflavone (Erythrina sigmoidea)	44.1 ± 4.9 (μM) (14.82±1.65μg/mL)	12.12	ND
	Eriodictyol (Vernonanthura tweedieana)	ND	ND	ND
Rocha et al 2019	Brachydin A (dimeric flavonoid)	>20 μM (>10.45 μg/mL)	ND	ND
	Brachydin B (dimeric flavonoid)	>20 (>10.73 μg/mL)	9.1	ND
	Brachydin C (dimeric flavonoid)	>20 (>10.13 μg/mL)	3.2	ND
Emiliano_&_Almeida-Amaral 2018	Apigenin	ND	ND	ND
Fonseca-Silva et al 2016	Apigenin	ND	ND	ND
Fonseca-Silva et al 2015	Apigenin	ND	ND	ND
Mai et al 2015	*G. oudiepe 1) 5,7-dihydroxy-3,3,4,6-tetramethoxyflavone*	ND	ND	ND
	2) 30,5,7-trihydroxy-3,4,5,6-tetramethoxyflavone	ND	ND	ND
	G. urvillei 3) 5,7-dihydroxy-3,3,4,5,6-pentamethoxyflavone	ND	ND	ND
	4) 5,7-dihydroxy-3,3,4,5,6-pentamethoxyflavone	ND	ND	ND
	5) 5,7-dihydroxy-3,3,4,5,6-pentamethoxyflavone	ND	ND	ND
	6) 40,5,7-trihydroxy-3,6,8-trimethoxyflavone	ND	ND	ND
	7) 40,5,7-trihydroxy-3,6-dimethoxyflavone	ND	ND	ND
	8) 5,7-dihydroxy-3,4,6-	ND	ND	ND

	trimethoxyflavone			
	9) 5,7-dihydroxy-3,4,6-trimethoxyflavone	ND	ND	ND
	10) 5,7-dihydroxy-3,4,6-trimethoxyflavone	ND	ND	ND
	11) Comercial kaempferol	ND	ND	ND
	12) 3-methoxy-kaempferol (kaempferol-3-monomethylether)	ND	ND	ND
	13) 3-methoxy-kaempferol (kaempferol-3-monomethylether)	ND	ND	ND
	14) 3-methoxy-kaempferol (kaempferol-3-monomethylether)	ND	ND	ND
	15) Semi-synthesis (triacetyl derivative)	ND	ND	ND
	16) Semi-synthesis (tetraacetyl derivative)	ND	ND	ND
Rizk et al 2014	*Selaginella sellowii* hydroethanolic extract (SSHE)	ND	Fibroblast cells (NIH/3T3) (12.2); Murine macrophages (J774.A1) (8.2)	ND
	Amentoflavone	ND	NIH/3T3 (22); J774.A1 (30)	ND
	Robustaflavone	ND	NIH/3T3 (9.1); J774.A1 (1.1)	ND
Assolini et al 2020	4-nitrochalcone (4NC) (comercial)	8.73 µM (2.21µg/mL)	2.1	0.41
	Kaempferol 7-*O*-methyl ether	>100 (µM) (>30.03 µg/mL)	ND	ND
	Kaempferol 3,7- di-*O*-methyl ether	>100 (µM) (>31.43 µg/mL)	ND	1.84
	Myricetin 3,7,3',4'-tetra-*O*	>100 (µM) (>36.03 µg/mL)	ND	ND
	Gossypetin 3,7,8,4'-penta-O-methyl ether	>100 (µM) (>33.23 µg/mL)	ND	ND
Dal Picolo et al 2014	Adunchalcone	53.71 ± 7.21 µM (27.21±3.65µg/mL)	ND	4.86
	Brachydin B	>20 µM (>10.73 µg/mL)	9.1	ND
	Brachydin C	>20 µM (10.13µg/mL)	3.2	ND
Lage et al 2013	quercetin 3-O-methyl ether	199.0 ± 25.9 µM (62.93±8.19µg/mL)	ND	10.4
	strychnobiflavone	125 ± 4.5 µM (78.81±2.84µg/mL)	ND	24.6
Manjolin et al 2013	Isoquercitrin	ND	ND	ND
	Quercitrin	ND	ND	ND
	7,8-dihydroxyflavone	ND	ND	ND
	Orientin	ND	ND	ND
	Isoorientin	ND	ND	ND

	Fisetin	ND	ND	ND
	Quercetin	ND	ND	ND
	Luteolin	ND	ND	ND
	Kaempferol	ND	ND	ND
	Galangin	ND	ND	ND
Gervazoni; Ozório and Amaral, 2018	2'-Hydroxyflavanone	88.15 ± µM (21,18 µg/mL)	28.5 (Wilt type) 26.2 (antimony resistent)	ND
Silva et al 2011	Quercetin	ND	ND	ND
Gontijo et al 2012	1) morelloflavone-4'''O-b-D-glycosyl	ND	ND	ND
	2) (±)-fukugiside	ND	ND	ND
	3) morelloflavone	0.29 µM (0.161µg/mL)	ND	ND
	4) Morelloflavone-7,4,7,3,4-penta-O-acetyl	>0.3800 µM (0.211µg/mL)	ND	ND
	5) Morelloflavone-7,4',7''',3''',4'''-penta-O-methyl	>0.3800 µM (0.211µg/mL)	ND	ND
	6) Morelloflavone-7,4',7''',3''',4'''-penta-O-butanoyl	>0.3800 µM (0.211µg/mL)	ND	ND
Grecco et al 2012	Naringenin	ND	ND	ND
	sakuranetin	39.50∗ (37.06–42.09) µg/mL	ND	ND
Salvador et al 2009	1) Pinostrobin	ND	ND	ND
	2) Pinocembrin	ND	ND	ND
	3) Tectochrysin	ND	ND	ND
	4) Galangin 3-methyl ether	ND	ND	ND
Lessa et al 2024	Naringenin	ND	ND	ND

Source: The autor

6.4 DISCUSSION

This study synthesizes key insights into the methodologies employed in *in vitro* research and highlights their implications for the development of alternative therapeutic strategies against *L. amazonensis*. Our review focused on the biological effects of plant-derived and propolis-based flavonoids on *L. amazonensis*, a protozoan parasite responsible for a form of CL prevalent in tropical regions.

Numerous natural compounds have been isolated from various parts of plants traditionally used in folk medicine to treat leishmaniasis (54–57). These findings emphasize the importance of fractionation techniques in identifying bioactive compounds and evaluating their potential therapeutic roles against *L. amazonensis*.

Our review identified over 30 compounds with activity against *L. amazonensis*, highlighting a wide array of bioactive compounds. In addition to plant-derived flavonoids, we found studies that investigated phenolic compounds isolated from propolis (58–60).

The key solvents used in the studies reviewed were hexane (FHVb), ethyl-acetate (FAEVb), and methanol (FMVb). This highlights the critical role of solvent selection in extracting bioactive chemicals from plants (56,57,60–62). The choice of solvent not only influences the chemical profile of the resulting extracts but also impacts their solubility and bioavailability in downstream assays.

Over 70% of the reviewed studies employed dimethyl sulfoxide (DMSO) as the primary solvent for diluting bioactive compounds prior to testing (63–66). Notably, around 20% of the reviewed articles did not use MTT or resazurin as their primary colorimetric methods for evaluating the antileishmanial activity of tested compounds (65,67–69). Instead, these studies adopted alternative techniques such as ATP quantification, flow cytometry, or direct microscopic counting, totaling 12 papers.

In *in vitro* assays, these compounds demonstrated varying levels of efficacy (Supplementary Table S3), with several showing promising antileishmanial activity while maintaining low cytotoxicity toward mammalian cells. Gontijo et al. (70) identified morelloflavone-4'''O-β-D-glycosyl, isolated from *Garcinia brasiliensis*, as the most active compound against both amastigote and promastigote forms, with an IC_{50} of 0.0234µg/mL. Salvador reported pinostrobin as the most active compound against amastigotes, with an IC_{50} of 0.0838µg/mL. Conversely, naringenin was the least effective compound against promastigotes, with an IC_{50} of 59.87 µg/mL. Sakuranetin showed moderate activity against amastigotes (IC_{50} = 51.89 µg/mL; 39.31 ± 69.98), based on the classification by Hassan et al. (4).

Another important parameter is SI, which considers both efficacy and cytotoxicity (Supplementary Tables S4). The flavonoid with the lowest SI value was luteolin (SI = 0.679), while carajurin presented the highest value (SI = 34.8).

Despite the high activity values observed for morelloflavone-4'''O-b-D-glycosyl and pinostrobin and the lower activity observed for naringenin, the SI alone was not used to support a deeper analysis of the treatment with these compounds. However, determining the CC_{50} is important for calculating SI values. Among the flavonoids reviewed, luteolin presented the lowest SI value (0.679) and carajurin the highest (34.8). Treatment efficiency can be assessed based on SI values, as an SI value greater than 10 indicates a compound with greater selectivity and promising potential for further investigation (71).

Although naringenin showed the lowest IC_{50} value for promastigote forms compared to other flavonoids analyzed in these studies—and even when compared to commercial drugs like miltefosine—this flavonoid also demonstrated potent *in vitro* effects against other *Leishmania* species, such as *L. donovani.* It activates CD4 and CD8 T cells, as well as Th1-type cytokines, which enhance the host immune response against the parasite. Moreover, it may reduce the side effects typically associated with commercial drugs due to its lower *in vitro* toxicity when used in monotherapy (72).

Another important aspect to highlight is the potential of drug combinations to improve treatment outcomes. Naringenin exhibited an additive effect with miltefosine against promastigote forms of *L. amazonensis*, reducing the dose of this compound by approximately twofold to achieve the same efficacy observed when the drug was used alone in *in vitro* assays (69).

Among the 52 studies analyzed in this review, only two presented FICI assays for promastigote forms and two for amastigote forms (Supplementary Table S2). This is an interesting topic to explore because the calculation of FICI allows investigation into the interactions of different flavonoids and drugs and opens the possibility of optimizing the treatment of leishmaniasis.

6.5 CONCLUSION

This review aimed to improve access to information by updating and summarizing recent research on flavonoid compounds against *L. amazonensis*. Flavonoids derived from natural sources, including plants and propolis, have demonstrated a wide range of activities against different forms of this species, with some exhibiting high levels of efficacy that could represent promising leads for the development of innovative, affordable drugs.

Most of the studies reviewed focused on the promastigote form of the *L. amazonensis*. *In vitro* assays remain crucial for screening extracts and isolated flavonoids, as well as for investigating their cellular and molecular mechanisms of action. This review highlights the relevance of studying natural components, especially flavonoids, as a strategy to advance our understanding and improve therapeutic approaches to *L. amazonensis* infection.

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7 CHAPTER II: *IN VITRO* EVALUATION OF THE COMBINATORIAL EFFECT OF NARINGENIN AND MILTEFOSINE AGAINST LEISHMANIA AMAZONENSIS

ABSTRACT

Leishmania amazonensis causes a clinical form called diffuse cutaneous leishmaniasis (DCL) with challenges to treatment, like low efficiency and drug toxicity. Therefore, it is necessary to investigate new therapies using less toxic leishmanicidal compounds, such as flavonoids like naringenin, and their combination with conventional drugs, such as miltefosine. Antileishmanial dose/response activity, isobologram, calculation of dose reduction index (DRI), and fractional inhibitory concentra tion index (FICI) tests were performed on *in vitro* assays using reference promastigote forms of L. amazonensis (IFLA/BR/67/PH8) to assess the combinatorial effect between naringenin and miltefosine. The *in vitro* results of isobologram, DRI, and FICI calculations showed that the combination of the compounds had an additive effect and was able to reduce the half maximal inhibitory concentration (IC_{50}) of miltefosine in the promastigote forms of the parasite compared to the treatment of the drug alone. This study demonstrated *in vitro* the viability of a combination action of the flavonoid with the treatment with miltefosine, opening space for further investigations on the association of natural compounds with the drugs used for the treatment of L. amazonensis.

Keywords: flavonoids; naringenin; n-hexadecylphosphonocholine; leishmaniasis; cutaneous leishmaniasis

7.1 INTRODUCTION

Leishmaniasis is a disease caused by single-celled protozoan of the genus *Leishma- nia*. The three main forms of the disease are visceral leishmaniasis (VL), mucocutaneous leishmaniasis (CML), and cutaneous leishmaniasis (CL). In the Americas, 18 countries are endemic to CL and CML, with Brazil, Colombia, Peru, Nicaragua, and Bolivia having the highest estimated case counts [1]. Among the species that cause CL in Brazil, *Leishmania amazonensis* has an incidence in primary and secondary forest areas of the legal Amazon (Amazonas, Pará, Rondônia, Tocantins,

and Maranhão) and also in the northeastern (Bahia), southeastern (Minas Gerais and São Paulo), central–western (Goiás), and southern (Paraná) states. This species causes a wide spectrum of clinical forms, which are localized cuta- neous leishmaniasis (LCL), CML, diffuse cutaneous leishmaniasis (DCL), and disseminated leishmaniasis (DL) [2].

Although DCL is relatively rare, it is extremely severe; diffuse skin infiltration and a large number of nodules and papules clinically characterize this form. Patients affected by DCL present lesions that cover the entire body, predominantly in the extremities, which rarely involve the nasopharyngeal mucous membranes [2]. Among the clinical forms caused by *L. amazonensis*, DCL is difficult to treat with conventional drugs; this clinical form has as its main characteristics the Th2 response with expression of interleukin-10, IL-4, and low expression of interferon gamma (IFN), leading to the patient's anergy to the parasite, high parasitic loads, and deforming lesions that generate physical stigmas and psychological impacts [3]. In addition to the ineffective immune response to DCL, the use of meglumine antimoniate and second-choice drugs like liposomal amphotericin B and pentamidine isethionate proved unsuccessful in cases of refractoriness [3]. Treatments with first- and second-choice drugs in the different clinical forms caused by *L. amazonensis* present some complications. These complications include toxic effects and restricted access of the population with economic vulnerability to medical care and follow-up, especially in the northern and northeastern Brazilian regions, due to the lack of resources and professionals. Additionally, drug resistance to treatment by meglumine antimoniate is an aggravating factor [4]. Miltefosine (n-hexadecylphosphonocholine), a phospholipid that is the hexdecyl monoester of phosphocholine with a molecular weight of 407.6 g/mol, shows a lower toxicity when compared with the meglumine antimoniate, easy oral administration, and an efficiency rate in the elimination of the parasite close to that of first- and second-choice drugs [5,6]. However, due to their teratogenic effect, new therapeutic alternatives demonstrating similar and less toxic efficacy have been explored. Efforts have been directed toward treatment based on plants and their metabolites, in particular the flavonoids, which have properties as preventive agents against cancer, antioxidant activity, and leishmanicidal activity [7,8]. Herein, we selected the flavonoid naringenin (5,7-Dihydroxy-2-(4-hydroxyphenyl) chroman-4- one), which is a trihydroxyflavanone, a flavanone substituted by hydroxyl groups at positions 5, 6, and 4 with molecular weight of 272.25 g/mol. In addition to its important immunomodulatory properties [5,9], naringerin has demonstrated potent *in vitro* activity against promastigotes and amastigotes of *Leishmania donovani* [10]. Considering the effects of naringenin and the

fact that miltefosine is the drug with less toxic effects, we sought to associate these two compounds and investigate their therapeutic potential against *L. amazonensis*.

7.2 RESULTS

7.2.1 *In Silico* Study

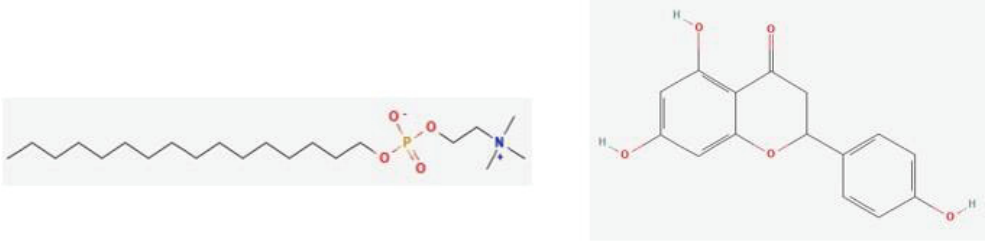
The physicochemical properties of naringenin and miltefosine were assessed to compare their predicted oral bioavailability using Lipinski's and Veber's criteria. Miltefosine presents one violation and naringenin none (Table 6). The structures of miltefosine and naringenin are represented in Figures 6a and 6b, respectively; for both substances, pan assay interference compounds (PAINS) were not identified by the SwissADME web tool.

Table 6. Molecular properties of naringenin and miltefosine according to Lipinski's and Veber's criteria and the number of pan assay interference compounds according to SwissADME. MW: molecular weight; Log Po/w: Log of partition coefficient (consensus LogP on SwissADME); RB: number of rotable bonds; H-Acc: number of hydrogen bond acceptors; H-Don: number of hydrogen bond donors; tPSA ($Å^2$): molecular polar surface area; PAINS: pan assay interference compounds.

	Naringenin	Miltefosine	Limit
MW	272.25 g/mol	407.57 g/mol	≤500
Log Po/w	1.84	3.35	≤5
RB	1	20	≤10
H-Acc	5	4	≤10
H-Don	3	0	≤5
tPSA ($Å^2$)	86.99 $Å^2$	68.4 $Å^2$	≤140
PAINS	0	0	-
Violations	0	1	-

Source: SwissADME web tool

FIGURE 4. THE STRUCTURES OF MILTEFOSINE AND NARINGENIN. (**A**) MILTEFOSINE PRESENTS A MOLECULAR FORMULA OF $C_{21}H_{46}NO_4P$ (PUBCHEM CID: 3599). (**B**) NARINGENIN PRESENTS A MOLECULAR FORMULA OF $C_{15}H_{12}O_5$ (PUBCHEM CID: 932)



(a) (b)

Source: Adapted from PUBCHEM [11]

7.2.2 Growth Curve

The growth of parasites presented an adaptation phase up to 48 h; after this period, the parasites reached their exponential growth phase in up to 96 h, reaching the stationary phase following 120 h of cultivation.

7.2.3 Antipromastigote Activity *In Vitro*

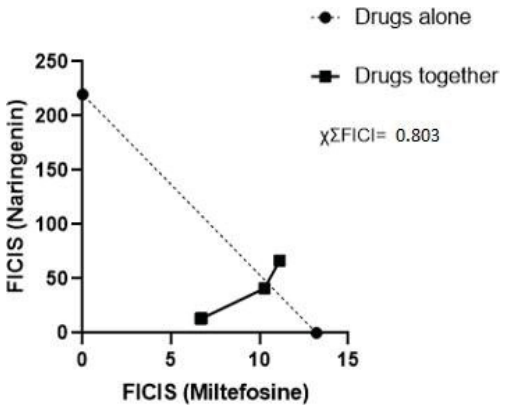
The mean IC_{50} obtained from the treatment with miltefosine alone was 13.20 µM, while naringenin had an IC_{50} of 219.86 µM. For the synergism tests, the IC_{50} values of the proportions 1:1, 2:1, 4:1, and 6:1 were first obtained, allowing the calculation of ΣFICI (Table 7) and enabling the construction of the isobologram (Figure 5). Calculating the average of the sum of FICI, we arrived at the value of χΣFICI, which was 0.803. It was observed that the interaction between the compounds is additive in all proportions tested.

Table 7. Representation of the IC_{50} values of miltefosine and naringenin in different proportions. FICI and DRI values were calculated using the formulas described in Section 4.3 of the Materials and Methods section. The IC_{50} values are represented with their mean and standard deviation; the FICI values are represented by the means extracted from the IC_{50} values. The DRI values are represented by means obtained by the calculation of the IC_{50} means of miltefosine in treatment alone and in combination with naringenin.

Proportion	IC_{50} Miltefosine (µM)	IC_{50} Naringenin (µM)	FICI	DRI
01:01	6.65 ± 0.9543	6.657 ± 0.9543	0.53	1.984277
02:01	6.73 ± 3.512	13.468 ± 7.029	0.57	1.961879
04:01	10.27 ± 3.832	41.08 ± 15.32	0.96	1.285955
06:01	11.12 ± 3.834	66.77 ± 23.00	1.14	1.186965
00:00	13.21 ± 3.125	219.9 ± 12.46		

Source: The autor

FIGURE 5. ISOBOLOGRAM ANALYZING THE INTERACTION OF MILTEFOSINE AND NARINGENIN



Description of the figure 5: Each point represents the FICI averages of the proportions 1:1, 2:1, 4:1, and 6:1 for naringenin and miltefosine; the points of the proportions 1:1 and 2:2 are overlapped due to the proximity of their FICI values. The dotted line represents the line of indifference. The result of χΣFICI is located at the top right.
Source: The autor

7.3 DISCUSSION

The compound combination strategy is adopted to optimize treatments for a wide range of diseases. Past studies have reported treatment regimens where antibiotics are combined to combat multidrug-resistant bacteria like *Acinetobacter baumannii*, reducing the mortality of infected patients [12], cancer cells such as breast cancer [13], and different types of viruses like HIV, HCV, and influenza [14]. The combination of compounds for treating leishmaniasis is also adopted to improve the effectiveness of treatments, diminishing their cost and time and reducing the likelihood of the emergence of resistant parasite strains. Compared to monotherapies, combined drug therapies present greater stimulation in the activity of leukocytes and their replication during infection, as well as increased production of cytokines that regulate the Th1 and ROS responses acting on the elimination of the parasite [15]. Faced with the challenges of monotherapies with first- and second-choice drugs, known by their side effects that hinder treatment, some efforts have been made to search for antiparasitic compounds in plants. In this sense, flavonoids stand out, which consist of a large group of phenolic compounds synthesized by the phenylpropanoid pathway in plants and have several antimicrobial, anti-cancer, and leishmanicidal activities [10,16]. In addition to treatments with isolated flavonoids, a previous study evaluated the optimization of the miltefosine treatment. When the drug was associated with the flavonoid apigenin, a reduction in parasitemia in mice was obtained with only half the dose of the drug when compared with the treatment of the drug alone [17]. As observed in the in silico analysis using the

SwissADME web tool, naringenin presents physicochemical properties that favor its bioavailability, opening space for further investigation of its leishmanicidal properties in isolation and in combination. We investigated the combinatorial effect of miltefosine with naringenin through using synergism and isobolagram testing to evaluate the interaction between the natural compound and the commercial drug to optimize the treatment of the drug, reducing its toxicity by associating it with a non-toxic natural compound [18]. The MTT assay performed herein showed values of the IC_{50} of the different proportions (Table 7). A reduction in miltefosine IC_{50} values was observed in all proportions; it was possible to assess that in the 1:1 and 2:1 proportions there was a substantial reduction in the IC_{50} dose, and to evaluate this reduction, the DRI calculation was used [19]. The DRI calculation measures how many times the dose of each drug in combination can be reduced due to the level of interaction up to a certain level of effect when compared to separate treatment. A DRI = 1 value indicates that there is no dose reduction; if the DRI > 1, it indicates a favorable dose reduction that leads to toxicity reduction; if the DRI < 1, the dose of the drug is not favorable for reduction. That is, it can be observed that in the proportions of 1:1, 2:1, and 4:1, they provided a reducing dose of miltefosine without reducing its effect, which, on the contrary, had a greater effect at lower doses than in the trial with the drug alone. The calculation of ΣFICI made it possible to construct the isobologram for better visualization of the interaction between the compounds. In Figure 5, it can be seen that the proportions of 1:1, 2:1, and 4:1 were below the indifference line with ΣFICI values of 0.53, 0.57, and 0.96, respectively. The 6:1 ratio presented a ΣFICI value of 1.16, slightly above the indifference line. According to the criteria for evaluating the interaction between compounds described in Section 4.3, it was concluded that the compounds in all proportions showed additive behavior ($1 > \Sigma FICI > 0.5$), with the ΣFICI values and the $\chi\Sigma FICI$ value of 0.803, defining the interaction between the compounds in general as additive. The additive effect between two drugs commonly refers to non-interaction or inertism between the substances being observed when the effect of the combination between different drugs is the sum of the effects of these drugs tested separately [19]. In general, it establishes a demarcation between the synergistic and antagonistic natures in the investigation of the interaction between drugs. Additive interactions have already been observed in several studies with combinations of leishmanicidal compounds [19–21]. The use of drugs with an additive effect in a combination can reduce chances of resistance, bringing the possi bility of shortening treatment time [20], making this

strategy attractive to mitigate the adverse effects present in the treatment of leishmaniasis. In summary, the combination of naringenin with miltefosine *in vitro* was able to potentiate the action of the drug, reducing the IC_{50} of the drug by approximately two times and requiring a lower dose of miltefosine to eliminate a considerable percentage of *L. amazonensis*.

7.4 MATERIALS AND METHODS

7.4.1 *In Silico* Study

The structures of naringenin and miltefosine were used to evaluate their theoretical physicochemical properties and the presence of PAIN. The predictions were calculated using the SwissADME web tool [22], considering Lipinski's rule of five (RO5) [23], followed by the additional rule proposed by Veber [24].

7.4.2 Growth Curve

Promastigote forms of the *L. amazonensis* strain (IFLA/BR/67/PH8) were maintained in an M199 medium with Hanks' salts (Sigma-Aldrich Brazil Ltd., São Paulo, Brazil) at 25 °C for inoculum production to be used in our experiments. The culture was maintained by replication every three to four days. The number of cells was measured in a hemocytometer and optical microscope, and the growth curve was performed in triplicate.

7.4.3 Antipromastigote Activity *in vitro*

The *in vitro* antipromastigote activity of miltefosine and naringenin was evaluated in promastigote forms of *L. amazonensis* using the 3-[4,5-dimethyl-thiazol-2-yl]- 2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich Brazil Ltd., São Paulo, Brazil) assay, with the drug tested alone and combined with naringenin [25] for a 48-h treatment. Commercial naringenin with a purity of 98% (Sigma-Aldrich Brazil Ltd., São Paulo, Brazil) and commercial miltefosine (Cayman Chemical, São Paulo, Brazil) were used, and stock solutions with concentrations of 50 mg/mL and 1 mg/mL were prepared in DMSO (Sigma-Aldrich Brazil Ltd., São Paulo, Brazil). We performed these tests to construct an isobologram to obtain the values of the FICI. The parasites were incubated at 25 °C for 48 h of treatment. After incubation, 50 µL of MTT

solution (10 mg/mL) was added to each well. The plates were maintained at 37 °C for 4 h. To solubilize the formazan crystals, 20 µL of 10% sodium dodecyl sulfate (SDS) (Sigma-Aldrich Brazil Ltd., São Paulo, Brazil) and 50 µL of 100% DMSO were added to each well. Plate readings were performed on a microplate reader using a wavelength of 550 nm. All tests were performed in biological triplicate and technical quintuplicate. The results are expressed as the compound concentration capable of inhibiting parasite growth by 50% (IC_{50}). The assay was performed in a 96-well flat-bottom microplate with a final volume of 200 µL. To perform the assay, the concentration of promastigote forms kept in the exponential phase was adjusted to 1×10^6 cells/mL. To calculate the IC_{50} of miltefosine alone, seven dilutions of the initial 200 µM solution of the drug were performed, where each new 2× dilution of the initial dose, the ranges of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM, and 1.5625 µM were obtained in the plate. In addition, an assay was performed with the isolated flavonoid. Following the same dilution strategy used for miltefosine but with different ranges of concentrations that are 1376.4 µM, 688.2 µM, 344.1 µM, 172.05 µM, 86.025 µM, 43.015 µM, 21.50 µM, and 10.75 µM, for these tests, the parasites were first seeded in the plate with M199 medium containing 0.75% DMSO with naringenin. After carrying out tests to evaluate the antipromastigote activity of the compounds in isolation, the next step was to carry out tests combining doses of naringenin and miltefosine in proportions of 1:1, 2:1, 4:1, and 6:1. With the IC_{50} values of the proportions, it was possible to evaluate the interactions of naringenin and miltefosine in the parasite's growth by calculating the fractional inhibitory concentration index (FICI) [26] and the dose reduction index (DRI) to evaluate the possibility of a decrease in the dosage of miltefosine in the combination assay without reducing its effect [19]. The isobologram was plotted with the sum of the FICIs and the averages of the sums of the FICI ratios, with ΣFICI calculated to determine the nature of the interaction between the natural compound and the drug, with $\Sigma FICI \geq 0.5$ indicating a synergistic effect, $0.5 > \Sigma FICI < 4$ additive effect, and $\Sigma FICI > 4$ antagonistic effect [27]. The formulas used to calculate the FICI and DRI are described below:

$$\Sigma FICI = \left[\frac{IC_{50}drug\ A\ combination}{IC_{50}drug\ A\ alone}\right] + \left[\frac{IC_{50}drug\ B\ combination}{IC_{50}\ drug\ B\ alone}\right] \quad (1)$$

$$DRI = \left(\frac{IC_{50}drug\ alone}{IC_{50}drug\ combination}\right) \quad (2)$$

7.4.4 Statistical Analysis

Statistical analysis and the construction of the isobologram was performed using GraphPad Prism 8.0.1 (GraphPad Software, Inc., San Diego, CA, USA). The results of the IC50 assays were transformed into log values and analyzed by a dose–response inhibition curve. A p-value ≤ 0.05 was considered statistically significant. The results were plotted with their means and standard deviations. The experiments were realized in biological triplicates and technical quintuplicates.

7.5 CONCLUSIONS

Although further work is needed, including tests with amastigotes, these results show that the association of naringenin with miltefosine has promising antileishmanial activity *in vitro* experiments, demonstrating that the flavonoid alone has an antipromastigote effect in *L. amazonensis* and can optimize the treatment of the commercial drug, increasing its efficiency.

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