Protease inhibitor activity of plant natural products as leishmanicine agents

Atividade de inibidores de proteases de produtos naturais contra agentes leishmanicidas

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ABSTRACT

Objective: Investigate plant natural products with inhibitory activity of *Leishmania* proteases, because the inhibition of certain proteases induce the parasite death. **Methods:** Descriptive/exploratory study, integrative review type. The search was performed in the following databases: Scientific Electronic Library OnLine (SciELO), Medical Literature Analysis and Retrieval System Online (MEDLINE), Latin American and Caribbean Health Science Literature Database (LILACS) and PUBMED. The descriptors were used in combination, from the

consultation in the Descriptors in Health Sciences (DECS) and Medical Subject Headings (MeSH), were: (*Leishmania*) AND (Protease Inhibitor) AND (Vegetable extracts); (*Leishmania*) AND (Protease Inhibitor) AND (Plant Extracts). Studies in English and Portuguese, published between 2000 and 2020, were included. Finally, the articles were categorized and analyzed. **Results and discussion:** This review included eight studies, published between 2000 and 2020. There were two publications in 2014, 2017 and 2019, in relation to the language, all eight studies were published in English. Five studies were carried out in India, two in Brazil and one in Iran. Seven of these were found in PUBMED database and one from MEDLINE, all were experimental, comprising *in vitro* research or using mice as animal models. **Conclusions:** It was noted that they use similar protocols for the isolation of protease inhibitor from plant species (affinity chromatography) and proteases from *Leishmania* sp. (cell lysis and protein precipitation and affinity chromatography). However, research involving Leishmania protease inhibitors are still scarce, requiring further studies on the subject, since the understanding of the functioning of these molecules has much to contribute to the development of new therapeutic targets.

Keywords: natural products, *leishmania*, proteases, protease inhibitor, leishmaniasis.

RESUMO

Objetivo: Investigar produtos naturais vegetais com atividade inibidora de proteases de Leishmania, pois a inibição de certas proteases induz a morte do parasita. **Método:** Estudo descritivo/exploratório, do tipo revisão integrativa. A busca foi realizada nas seguintes bases de dados: Scientific Electronic Library OnLine (SciELO), Medical Literature Analysis and Retrieval System Online (MEDLINE), Latin American and Caribbean Health Science Literature Database (LILACS) e PUBMED. Os descritores utilizados em combinação, a partir da consulta nos Descritores em Ciências da Saúde (DECS) e Medical Subject Headings (MeSH), foram: (Leishmania) AND (Protease Inhibitor) AND (Extratos Vegetais); (Leishmania) AND (Inibidor de Protease) AND (Extratos Vegetais). Foram incluídos estudos em inglês e português, publicados entre 2000 e 2020. Por fim, os artigos foram categorizados e analisados. **Resultados e discussão:** Esta revisão incluiu oito estudos, publicados entre 2000 e 2020. Houve duas publicações em 2014, 2017 e 2019, em relação ao idioma, todos os oito estudos foram publicados em inglês. Cinco estudos foram realizados na Índia, dois no Brasil e um no Irã. Sete deles foram encontrados no banco de dados PUBMED e um no MEDLINE, todos experimentais, compreendendo pesquisas in vitro ou usando camundongos como modelos animais. **Conclusões**: Notou-se que os estudos utilizam protocolos semelhantes para o isolamento de inibidor de protease extraídos das plantas (cromatografia de afinidade) e proteases de Leishmania sp. (lise celular e precipitação de proteínas e cromatografia de afinidade). No entanto, pesquisas envolvendo inibidores de proteases de Leishmania ainda são escassas, necessitando de mais estudos sobre o assunto, pois o entendimento do funcionamento dessas moléculas tem muito a contribuir para o desenvolvimento de novos alvos terapêuticos.

Palavras-chave: produtos naturais, *leishmania*, proteases, inibidor de protease, leishmaniose.

1 INTRODUCTION

Leishmaniasis are chronic infectious diseases caused by species of obligate intracellular protozoa from *Leishmania* genus (Trypanosomatidae family and Kinetoplastida order), which generally, are transmitted by vectors insects to mammalian hosts [1]. The parasite can also be transmitted by transplacental, blood transfusion and through contaminated needles [2]. They are endemic in 92 countries from tropical and subtropical areas in the Old and New World, affecting poor populations living in precarious sanitary conditions. There are believed to be 68 million people living at risk for these diseases, 12 million infected individuals and an estimated 700,000 to 1 million cases annually. Leishmaniasis is classified as one of the most neglected tropical diseases, with high morbidity and low mortality [3].

Currently, about 54 species of *Leishmania* genus have been reported, and at least 21 of them are pathogenic to humans [4]. Promastigote parasites are transmitted through the bites of infected female sandflies, which feed on blood to produce eggs. Approximately 70 animal species, including canids, rodents, marsupials, mongooses, bats, cats, hyraxes, and humans, have been found as natural reservoir hosts of *Leishmania* parasites, therefore, the disease can be classified as zoonoses, anthropozoonosis or anthroponese, although few species are strictly anthroponotic [5].

Protozoan parasites from *Leishmania* genus have a digenetic life cycle. They live alternately into vertebrate hosts and insect vectors (Psichodidae family, Phlebotominae subfamily, *Phlebotomus* and *Sergentomyia* genera in the Old World and *Lutzomyia*, *Brumptomyia*, and *Warileya* in the New World), and exhibit two morphological forms: the extracellular promastigote form is motile, elongated with free flagellum and is found into the digestive tract of sandfly, and the amastigotes intracellular form, that is rounded, smaller with non-exteriorized flagellum that infect lysosomal vacuoles in phagocytic cells [6,7]. In the digestive tract of vector, promastigotes transform themselves in the non-dividing, infectious ‘metacyclic’ promastigotes that are transmitted by sandfly bite. These promastigotes are phagocyted by phagocytes, such as macrophages, and inside these cells they survive, multiply, cause the lysis of the host cells, releasing amastigotes that infect neighboring macrophages [2].

The clinical manifestations of leishmaniasis range from the self-healing cutaneous (CL), the mucocutaneous skin ulcers (MCL) and the diffuse cutaneous (DCL) in cellular-mediated immune response deficient hosts to the lethal visceral (VL) form (visceral leishmaniasis or kala-azar) and postkala-azar dermal leishmaniasis [8,9]. This clinical

spectrum is related to *Leishmania* species that is involved. The epidemiology of these diseases depends on the characteristics of the parasite and sandfly species, the local ecological characteristics of the transmission sites, current and past exposure of the human population to the parasite, and human behaviour [2,9,10].

The leishmaniasis-related disabilities impose a great social burden, and reduce the economic productivity in endemic areas. It has also been observed that infected people with MCL live in isolation because to the social stigma of deformities and disfigurement scars [2]. Furthermore, *Leishmania*–HIV co-infection have been observed in areas where both diseases are endemic, and are associated to high mortality, because the low response to chemotheraphy representing an important challenge in the public health [11].

1.1 LEISHMANIASIS TREATMENT

There are no approved and available vaccines for human leishmaniasis. Thus, programs of prevention that include individual protection (protective nets and repellents), vector control (inseticides), and chemotherapy are the main mechanisms to handle this disease [12].

Since 1940s, pentavalent antimony compounds, for example Glucantime and Pentostam, or branded other formulations, have been the mainstays of antileishmanial therapy. Although they have good therapeutic index, their administration is parenteral with high dosages many doses a day, consequently they induce severe side-effects, such as myalgia, abdominal pain, liver, heart and kidney changes, the patients give up the treatment, and drug resistance has also been observed [2,13]. Besides, the parasite persists in the scars of clinically cured patients [14]. The treatment of leishmaniasis is very expensive when compared with other tropical diseases, contributing to the worsening of the disease [15].

In all parts of the world, including in Brazil, Glucantime is the first choice drug, being effective for CL, MCL and LV because the success in regressing the clinical and hematological manifestations of parasite infection [16]. Besides, this drug has fast excretion within 48 hours by the kidneys it is necessary to administer high doses to obtain efficacy in the treatment, but this can induce to the patient's death [17, 18].

The mechanism of pentavalent antimony compounds action is the inhibition of glycolysis and the β-oxidation enzymes of *Leishmania*. However, antimony is a heavy metal, it is not a selective drug and interfere with other metabolic pathways of parasites and hosts. Furthermore, these drugs can interact with the zinc finger domain of proteins,

and many proteins have this motif in their tridimensional structures, explaining the high toxicity of these compounds [2,12,13].

The leishmaniasis second-line drugs are employed in endemic areas with high rates of unresponsiveness to antimonial chemotheraphy. They are pentamidine, amphotericin B, allopurinol, and more recently, miltefosine, paramomicine and sitamaquine [19]. However, they are more toxic, expensive, and have low therapeutic index when compared to antimony compounds. Pentamidine an amphotericin B are the most employed. Pentamidine is administrated in cases of non-response to antimonials or patients with VL, but it has important restrictions due to the high toxicity, that include hypoglycemia, hypotension, cardiac alterations, nephrotoxicity and even sudden death [20]. Amphotericin B was previously used as an antifungal, but it has been used in the treatment of MCL due to the expressive leishmanice effect [21]. However, it induced several adverse effects such as seizures, chills, fever, anemia, anorexia, and decreased kidney function. This antifungal is incorporated into the macrophage, killing the *Leishmania* [22].Their toxic effects are caused by the interaction with ergosterol and episterol in parasites membranes, and the binding to cholesterol present in the host's cells plasma membrane [23]. To reduce these side effects, three formulations were developed to be release inside the infected cells: liposomal amphotericin B, lipid complex amphotericin B and colloidal dispersion amphotericin B. Clinical studies for VL treatment demonstrated good tolerability and no signs of renal and hepatic toxicity [24, 25, 26]. The major disadvantage of these formulations is the high cost, restrict the use in public health services [27].

More recent drugs, as miltefosine showed to be ineffective, and considered harmful to patients due to the gastrointestinal toxicity. Besides, the low sensitivity to some species of *Leishmania* in some geographic regions, restricted its use [28]. Paramomycin when combined with antimonials reduces the duration of therapy, and has the advantage of fighting coinfections, but it can cause nephrotoxicity and damage to the eighth cranial nerve [29].

1.2 *LEISHMANIA* PROTEASES AS THERAPEUTIC TARGETS

The target identification is one of the most importat step in the rational drug development. It should be absent, or different from the host homolog, in order to be exploited as a drug target. *Leishmania* biochemical pathways that guarantee the parasite survival, proliferation, and infection are targets that have been investigated. They are

enzymes that regulate or participate in biosynthesis of sterol, hypusine, folate, and glycosylphosphatidylinositol; glycolysis; purine salvage; glyoxalase and trypanothione systems, or special enzymes such as protein kinases, topoisomerases and proteases [2,30].

Proteases or peptidases catalyze the cleavage of peptide bonds in proteins and peptides, originating peptides of various sizes and free aminoacid [31]. They are found in all organisms, organs and organelles, and participate in many essential physiological processes, such as amino acid assimilation, cell death, differentiation, digestion of extracellular matrix, growth of tissues and organs, and in microrganisms they are important virulence factors [2,32].

These enzymes are classified according to the catalyc aminoacid ou a metal in the active site: serine, cysteine, aspartic, treonine, glutamic, asparagine, and metaloproteases [33]. In *Leishmania* were described cysteine, serine, aspartic, treonine, and metaloproteases [34].

Several studies indicated that *Leishmania* proteases are involved in the invasion of host tissues, differentiation in the parasite's life cycle, proliferation and growth, modulation and escape from the host's immune system, nutrition, metabolism of biologically active proteins or peptides, survival within macrophages, signaling, paths of death and sustain the process of infectious diseases, or even in the parasite's resistance to drug therapy. Thus, *Leishmania* proteases are important virulence factors, and potential therapeutic targets for the leishmaniasis treatment [2,12,32].

Cysteine proteases is the best-characterized group enzymes in *Leishmania* and they have their sequences in databases and genes isolated. These enzymes belong to Clan CA and the papain-like protease superfamily. These parasites express a broad range of cysteine proteases (CPs), and the best characterized of which are CPA, CPB, and CPC (CPs A, B, and C). CPA and CPB are cathepsin L-like and show some functional redundancy, while CPC is cathepsin B-like [35]. Inhibitors of *Leishmania* CPs, indicated that these enzymes are involved in macrophages infection, amastigotes survival in these host cells, as well as they are modulators of the host's immune response, suggesting that these CPs are virulence factors of these parasites [36,37,38].

Many compounds such as, vinyl sulfone, dihydrazide, palladacycle, and organotellurane, have showed success *in vivo* treatment of CL and VL by CPC inhibition [39], and *in vitro* assays identified multiple compound classes active against CPB (e.g., semicarbazones, thiosemicarbazones, triazine nitriles [40], and benzophenones [41]). Although CPs inhibitors seems to be promising, the activity of CPA, CPB, and CPC

families would be blocked to prevent parasite invasion and replication in host cells. Non-selective inhibitors can also inhibit the host CPs.

Another enzyme already described in *Leishmania* is the metalloprotease, called glycoprotein 63 (gp63) or leishmanolysin, being described as the largest antigen expressed on the surface of the promastigote form of several species. This enzyme is one of the main surface components present in all species of the *Leishmania* genus, especially in the promastigote forms, representing more than 1% of the total parasite proteins [42].

Studies demonstrate that gp63 has a protective activity against *Leishmania* degradation by the phagolysosome. In another study, decreased survival rate of attenuated strains was associated with a 20-50% reduction in *Leishmania* gp63 expression. In another study, it was shown that proteins incorporated in liposomes coated with *L. mexicana* gp63 were protected from the phagocytic action of macrophages. After gp63 denaturation, phagocytic action was observed, thus corroborating the protective action of gp63 in phagocytosis [43, 44].

Therefore, proteases are important virulence factors for the parasite, ensuring the survival and maintenance of infection by the parasite, being those of the serine, metal, aspartic, threonine and cysteine types described in Leishmania, and among these, the most studied proteases in Leishmania are of the cysteine class. And the inhibition of these proteases by specific inhibitors can interfere with the development of leishmaniasis, thus being a potential therapeutic alternative.

1.3 PLANT PROTEASE INHIBITORS

Many plants are used by traditional communities, and in many developing countries a large part of the population depends on the use of plants to treat their diseases [47]. Based on this knowledge of popular use, studies are carried out with plant extracts from different families or with metabolites from the most diverse chemical classes isolated from these extracts, among these classes, many perform anti-*Leishmania* activity, such as protease inhibitors.

Proteases and protease inhibitors (PIs) are a class of proteins participating in the biochemical duel between plants, phytopathogenic microorganisms and insect pests [47, 40]. They act as regulatory agents for proteolysis in several organisms and in plants in particular, constituting important defense strategies against predators and pathogens, since plants do not have an immune system [48].

In plants, the first report on a protease inhibitor was made by Read and Haas, in 1938 [49]. The first works were related to animal nutrition. Due to the deleterious effects of this category of proteins found in many plants used in the diet of animals for slaughter, they initially became known as anti-nutritional factors [43].

The first well-characterized inhibitor was soybean trypsin (KUNITZ, 1947) [50], which was isolated, crystallized and complexed with pig trypsin, being the first classical methodological model for the biochemistry of protease inhibitors. Shortly thereafter, the first study of plant protease inhibitors was done by Borchers and Ackerson (1947) [51, 52].

Until 1976, only one highly specific inhibitor for a microbial protease had been well isolated from plants, the subtilisin inhibitor, from barley. In the same year, it was observed that trypsin and chymotrypsin inhibitors present in soybean and bean seeds and also in potatoes were able to suppress the activity of proteases secreted by *Fusarium solani* (Mart.) Sacc. [53].

Similar results were later obtained on the action of other PIs on extracellular enzymes, growth and development of phytopathogenic microorganisms [40].

Therefore, PIs in plants may be able to suppress the enzymatic activity of phytopathogenic microorganisms. These inhibitors can also be synthesized constitutively or the synthesis induced in response to attack by external agents [47]. Thus, the present work aimed to raise information about plant extracts active against *Leishmania* proteases [30, 54, 55].

2 METHODS

This research is a descriptive/exploratory study, of the integrative review type. Therefore, the present review was organized following the Prisma recommendation (Preferred Reporting Items for Systematic Reviews and Meta-Analyses), with the elaboration of a four-step flowchart, which are described below and illustrated in Figure 1. The Prisma recommendation consists of a checklist with 27 items and a four-step flowchart, allowing both to improve the reporting of systematic reviews and meta-analyses and to assist in the critique of published systematic reviews [56].

Step 1 – the search for articles was performed in the following databases: Scientific Electronic Library OnLine (SciELO), Online Medical Literature Analysis and Retrieval System (MEDLINE), Latin American and Caribbean Health Science Literature Database (LILACS) and PUBMED. The descriptors were used in combination, from the

consultation in the Descriptors in Health Sciences (DECS) and Medical Subject Headings (MeSH), were: (Leishmania) AND (Protease Inhibitor) AND (Vegetable extracts); (Leishmania) AND (Protease Inhibitor) AND (Plant Extracts).

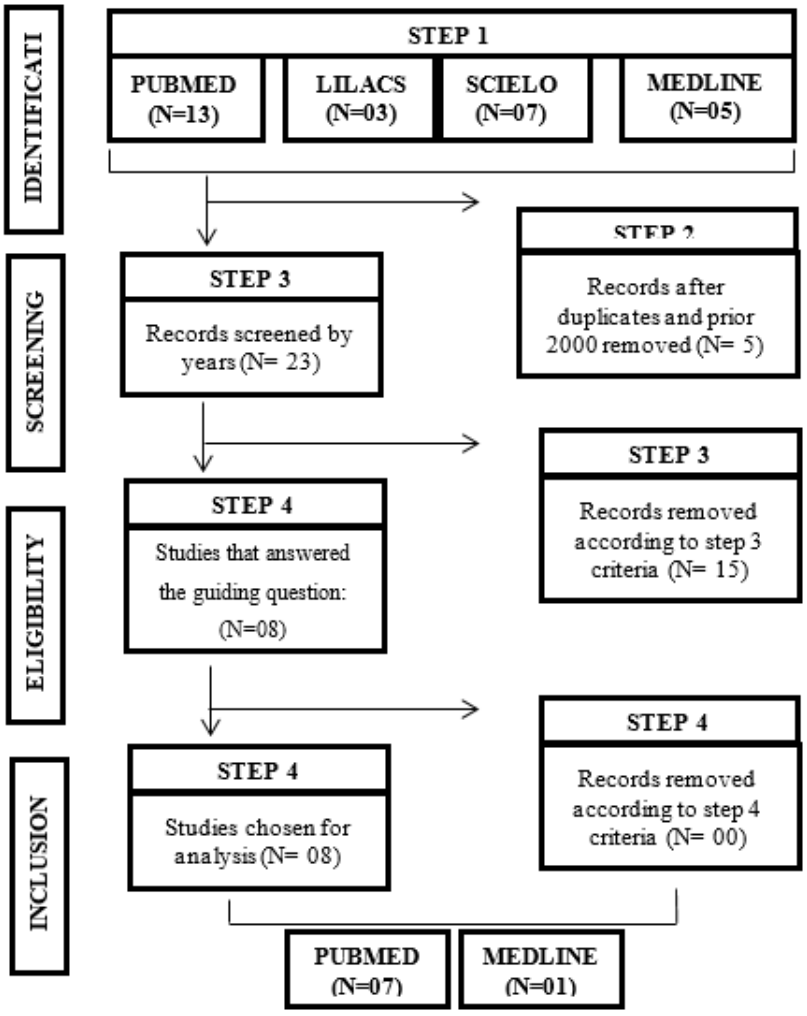
Step 2 - was read the titles to check overlapping of studies between the lifts of the four databases and for deletion of articles prior to 2000.

Step 3 – Articles in English, Portuguese and Spanish, published between 2000 and 2021, in article format and available in full, were included. Which obeyed the following guiding question: "Which plant extracts are active against leishmania proteases?"

Previous articles, theses, dissertations, technical reports, reviews, book chapters, editorials, letters to the editor and newspaper articles were excluded.

Step 4 – finally, the selected articles were read in full for further categorization and analysis by year, journal, database, language and location of the study, after having applied all the eligibility criteria.

Figure 1. Flowchart of article selection, according to the PRISMA method, 2020



Source: Authors, 2022.

3 RESULTS AND DISCUSSION

Eight studies were included, published between 2000 and 2020. With more publications in 2019 and 2014, with two publications each, all studies were in English, five studies were carried out in India and two in Brazil and one in Iran, as the basis of data, seven studies were from PUBMED and one from MEDLINE, all experimental studies, *in vitro* or *in vivo* (Table 1). The synthesis of the studies was also carried out, regarding the objective, type of *Leishmania*, evolutionary form, plant/plant part, type of extract and type of protease inhibitor in *Leishmania* (Table 2).

Table 1. Description of the articles used in ths study, according to the journal, year, databases, languages, places of study and types of study.

Journal	**Year**	**Data base**	**Country**
Experimental Parasitology	2017	PubMed	India
Biomedicina & Pharmacotherapy	2019	PubMed	India
Journal of Global Antimicrobial Resistance	2019	PubMed	Iran
International Immuno-pharmacology	2020	PubMed	India
Journal of Medicinal Food	2011	PubMed	Brazil
Experimental Parasitology	2014	Pubmed	India
Current microbiology	2017	Pubmed	India
BioMed Research International	2014	MEDLINE	Brazil

Source: Authors, 2022.

Table 2: Synthesis of studies according to Objective; *Leishmania* species; Evolutionary form; Plant/plant part; Type of extract; and Target enzyme.

Reference	Objeticve	*Leishmania species*	Evolutionary form	Plant/ Vegetable Part	Extract	Target enzyme
Rodrigues et al., (2014) [67]	To evaluate the anti-*Leishmania* effects of the hexanic extract of *Arrabidaea chica* (Humb. & Bonpl.) Verlot leaves	*L. amazonensis* *L. infantum*	Promastigote	*Arrabidaea chica* (HBK) Verlot (leaves)	Hexanic	Peptidase
Inácio Filho (2018) [69]	To study the effect of EGCG (epigalocatechin-3-gallate) *in vitro* and *in vivo*, its associations and molecular mechanism of action in *Leishmania infantum*	*L. infantum*	Promastigote Amastigote	Green tea (epigallocatechin-3-gallate)	Ethanolic	Trypanothione Reductase
Paik et al., (2014) [62]	To evaluate the anti-*Leishmania* potential of the fraction of crude extract from *Solanum tuberosum* (L.), rich in serine protease inhibitors, targeting *Leishmania* serine proteases	*L. donovani*	Promastigote	Tuber of *Solanum tuberosum* (L.)	Ethanolic	Serine protease
Pereira et al., (2011) [58]	To evaluation of *Leishmania* protease inhibitory activity of hexanic, ethyl acetate and ethanol extracts; and bioflavonoid fukugetin, from the pericarp of the fruit of *Garcinia brasiliensis* (Mart).	*L. amazonensis*	Amastigote	*Garcinia brasiliensis* (pericarp)	Ethyl acetate	Protease
Das et al., (2015) [76]	To evaluate the anti-*Leishmania* activity *in vitro* of Cg-Ex, targeting the *Leishmania donovani* serine protease(s).	*L. donovani*	Promastigote	*Coccinia grandis* (L.) Voigt (leaves)	Ethanolic	Serine protease
Paik et al., (2016) [63]	To determine the anti-*Leishmania* potential of the serine protease inhibitor obtained from the crude potato tuber extract in an *in vivo* model	*L. donovani*	Amastigote	Tuber of *Solanum tuberosum* (L.)	Ethanolic	Serine protease
Pramanik et al., (2017) [80]	To investigate and characterize the anti-*Leishmania* efficacy of Coccinia grandis (L.) Voigt leaf extract (Cg-Ex) with its immunomodulatory property against *Leishmania donovani* in an *in vitro* experimental model.	*L. donovani*	Promastigote	*Coccinia grandis* (L.) Voigt (leaves)	Ethanolic	Serine protease
Dutta et al., (2007) [84]	To evaluate the supposed leishmanicidal action mechanism of Aloe vera (L.).	*Leishmania donovani*	Promastigote	*Aloe vera* (L.) Burm.f. (leaves)	Ethanolic	Serine protease

Source: Authors, 2022.

3.1 *GARCINIA BRASILIENSIS* MART

Garcinia brasiliensis Mart., also known as *Rheedia brasiliensis* Planch & Triana, is cultivated throughout the Brazilian territory, being popularly known as bacuri, bacupari, porocó and bacuripari in Brazil, and as guapomo in Bolivia. It is a species native to Brazil, Paraguay and northern Argentina [57].

In a study by Pereira (2009) [55], the leishmanicidal action of the hexanic extract of *G. brasiliensis* was investigated, and the extract presented IC50 values of 1.43 µg/mL and 10.66 µg/mL in promastigotes and amastigotes in infected macrophages, respectively.

Pereira et al., (2011) [58] used the compound fukugetin, a bioflavonoid, which was purified from the extract of ethyl acetate from the pericarp of the fruit of *G. brasileisnsis*. The purification method was established by Derogis et al (2008) [59].

After isolated, the bioflavonoid fukugetin was tested against *Leishmania* proteases, which were obtained from *L. amazonensis* amastigotes, which were isolated from infected rats. The cells were washed and centrifuged, after the parasite lysates, the samples went through the ultrasonication process, were again centrifuged and the supernatants containing the proteases were used in the test [58].

And, after evaluating this bioflavonoid against *Leishmania* proteases, it showed a greater inhibitory effect than ethyl acetate extract, with an IC50 of 5.2 - 0.5 µM / mL.

These data indicate that fukugetin is a potent protease inhibitor of *L. amazonensis*, and that it also did not cause toxicity in mammals or in mammalian cells *in vitro*, thus, the study by Pereira et al., (2011) [58] points out new perspectives on the development of drugs with leishmanicidal action, obtained from natural products that target the parasite's proteases.

3.2 *SOLANUM TUBEROSUM* (TUBER FROM POTATO)

Solanum tuberosum L. is a valuable plant, non-toxic and highly nutritious consumable [60]. This plant has animal pancreatic proteinase inhibitors and potato tuber soluble proteins have a mixture of chymotrypsin, trypsin, elastase and carboxypeptide inhibitors [60, 61].

Paik et al., (2014) [62] isolated a fraction rich in serine protease inhibitors obtained from potato tuber, through the reverse zymography method, used for serine protease inhibitors, standardized by Hanspal et al (1983).

For the isolation of proteases, the *L. donovani* metacyclic promastigotes were centrifuged, then lysed using a protocol with several cycles of freezing and thawing, after which the lysate was centrifuged and the supernatant was collected and dialyzed, and centrifuged again, and had its enzymatic activity evaluated through the gelatin zymogram. The gel electrophoresis of the substrate was performed, and after that the gel was incubated in the presence and absence of potato tuber serine protease inhibitor and aprotinin, used as a positive control [62].

The results of Paik et al (2014) [62] showed that the activity of the serine protease from *L. donovani* was inhibited by the rich fraction of potato tuber inhibitors (0 – 2.5 mg/mL), in a dose-dependent manner. The same inhibitor was tested at a higher concentration, and it significantly inhibited the *Leishmania* serine protease activity when compared to the classic serine protease inhibitor, aprotinin.

When the assay was performed using the substrates BApNA and BTpNA, it was shown that the activity of commercial proteases trypsin and chymotrypsin, and Leishmania protease, the activity of these enzymes was reduced according to the increase in the concentration of potato turbulent inhibitor, however, this inhibitor exhibited stronger inhibition against commercial trypsin and serine protease from *Leishmania*.

The inhibitor was also tested in promastigotes through the *in vitro* MTT assay. The inhibitor showed significant inhibitory activity in *L. donovani* promastigotes and amastigotes incubated with variable concentration (0–2.5 mg/ml) for 48 h, and inhibitory concentration (IC50) of 312.5±0.1 µg/mL and IC50 82.3 ± 0.2 µg/mL, respectively [62].

In 2016, Paik et al., (2016) [63] analyzed this same rich fraction in serine protease inhibitors, isolated from the ethyl acetate fraction of potato tuber extract in an *in vivo* model. And to verify the anti-*Leishmania* action, BALB/c mice were treated for thirty days with the potato tuber inhibitor, orally, divided into two groups, at dosages of 12.5 mg/kg (Group 1) and 25 mg /kg (Group 2). When analyzing the liver smears, it was noted that a marked decrease in the parasite load of the treated animals. The parasite load in Group 1 showed a significant decrease ($p < 0.05$) in the number of amastigotes, reducing 86.9% in the liver and 88.7% in the spleen, when compared to the infected controls.

In another study, also carried out by Paik et al., (2020) [64], the fraction rich in serine protease inhibitor obtained from potato was analyzed by sephadex column chromatography and the fractions with higher absorbances were collected (PTF1, PTF2 and PTF3), and the inhibitors identified by reverse zymography.

The three fractions (PTF1, PTF2 and PTF3) were tested against L. donovani serine protease, through the MTT method, to determine the inhibitory concentration of 50% of the parasites. When analyzing the IC50, the PTF1 and PTF3 fraction showed IC50 of 382.6 ± 1.2 µg / ml and 143.5 ± 2.4 µg / ml, respectively ($p < 0.05$), and the PTF2 fraction was considered less efficient in killing promastigotes. And when comparing the PTF1 and PTF3 fractions, it was found that there was 90% growth inhibition by PTF3, being more effective than the PTF1 fraction. The authors also analyzed the morphology of the parasites treated with the PTF3 fraction, compared to the parasites treated with miltefosine, which showed irregularity in morphology, similar to that of the parasites treated with miltefosine.

The PTF3 fraction was also evaluated in murine macrophages, and exhibited minimal cytotoxicity of 11.3%, and when analyzing the selectivity index, the selectivity of the PTF3 fraction was very high compared to the other fractions (PTF1 and PTF2). And when the parasite load was evaluated, it was reduced to 82.3 ± 0.9%, at a concentration of 200 µg/ml, in 24 hours, and after 48 hours the PTF3 fraction showed a reduction of 94.1 ± 0.8 % of the parasite load.

Data from Paik et al., (2020) [64] indicate that the PTF3 fraction is effective in reducing the parasite. It was also evidenced that the PTF3 fraction significantly inhibited the serine protease of *L. donovani*, at the concentration of 0.5 mg/ml. And when incubated with PTF3 and aprotinin, there was a decrease of 83.1 ± 1.7% in the infection rate.

And when tested in BALB/c mice, at a dose of 23 mg/kg, the PTF3 fraction almost eradicated the infection, decreasing the parasite load in the liver and spleen by 89.3 ± 0.1% and 88.5 ± 0. 5%, respectively.

The studies by Paik et al., (2014; 2016; 2020) [62, 63, 64] showed a potential leishmanicidal effect of the serine protease inhibitor in both *in vitro* and *in vivo* models. And as this inhibitor was considered less toxic to host cells, it is interesting to be studied as a potential therapeutic agent against leishmaniasis.

3.3 *ARRABIDAEA CHICA* VERLOT

Arrabidaea chica Verlot, popularly known as pariri, belongs to the Bignoniaceae family. It is endemic in almost all over Brazilian territory, being found most frequently in the Amazon rainforest, where it is used to treat skin diseases, anemia, jaundice and inflammatory reactions [45, 65].

In a study carried out with the crude extract of *A. chica* leaves against *L. amazonensis* promastigotes, the IC50 was determined at a concentration of 155.9 µg/mL. The cytotoxicity test was also performed on macrophages of the J774.G8 lineage, over a period of 24 hours, and the concentration that demonstrated toxicity in 50% of the cells was 189.9 µg/mL, corresponding to CC50 [65].

The extract of *A. chica* was also fractionated in silica column chromatography, resulting in five fractions (B1, B2, B3, B4 and B5), and when tested on the parasite, the B2 fraction was more active with IC50 of 37.2 and 18.6 *µ*g/mL against peptidases from *L. infantum* and L. *amazonensis*, respectively.

To perform the *L. amazonensis* and *L. infantum* peptidase inhibition assay, the promastigotes were washed and centrifuged, going through several cycles of freezing and thawing, and then the cell extracts were centrifuged and the supernatants were preserved. And to analyze the peptidase activity (gelatinase), was used the protocol described by Cedrola et al., (2012) [66].

Rodrigues et al., (2014) [67] also evaluated the effect of the B2 fraction on metalloproteinases from *L. infantum* and *L. amazonensis* lysates, however, even with the decrease in enzymatic activity, the results showed that the B2 fraction was less effective against this class of enzymes.

Furthermore, the study evidenced that the B2 fraction completely inhibited the activity of promastigotes, thus, the results indicate that the use of *A. chica* is an interesting source of *Leishmania* protease inhibitors, since studies have also shown that the compounds extracted from the leaves of *A. chica* are potential leishmanicidal agents, in non-cytotoxic concentrations [65, 67].

3.4 GREEN TEA

Green tea is a type of tea made from the infusion of the *Camellia sinensis* plant, which belongs to the Theaceae family (Ternstroemiaceae), and is popularly known as tea from India, tea tree or tea tree. Green tea contains biologically active compounds such as polyphenols, methylxanthines and essential oils. Most of its biological actions, such as the reduction of plasma lipid levels, anti-inflammatory effects, antimicrobial, antineoplastic and antioxidant activities, are related to the polyphenol fraction, especially catechins [68].

In a study carried out with Epigallocatechin-3-gallate (EGCG), a compound present in green tea, the *in vitro* and *in vivo* effect and the molecular mechanism of action

with the involvement of trypanothione reductase (TR) in *Leishmania infantum* were analyzed [68]. TR is a flavoenzyme that is mainly present in the Trypanosomatidae family. This is a key molecule for redox metabolism in trypanosomatids, providing adequate conditions for the survival of the parasite.

Inácio Filho (2018) [69] demonstrated that EGCG has anti-promastigote activity with an IC50 of 162.0 µM, and IC90 of 262.5 µM and anti-amastigote activity with an IC50 of 3.8 µM with a 90% inhibition in the concentration of 24µM. As for the in vivo result, the compound significantly reduced the number of parasites by 92% at the highest dose administered (50mg/kg/day), demonstrating ED50 and ED90 values of 7mg/kg and 9.9mg/kg, respectively [68].

To analyze the ability of EGCG inhibition against trypanothione reductase, an enzymatic assay was performed based on the Elman method (HAMILTON et al., 2003) [70] which is based on the reduction of DTNB [5,5'-dithio acid -bis-(2-nitrobenzoic)] to 2TNB (2-nitro-5-thiobenzoic acid) by reduced trypanothione [T(SH)2], leading to oxidized trypanothione [T(S)2] which will be regenerated to T(SH)2 by TR [71]; and also through molecular docking.

In the trypanothione reductase (TR) inhibition assay, EGCG significantly inhibited TR, demonstrating a dose-dependent profile, with an inhibition of approximately 65% compared to the untreated control. This result may be related to the production of reactive oxygen species, since the author states that the compound may have inhibited the key enzyme in the redox balance of these parasites, thus decreasing the TR activity [69]. Furthermore, several works report the inhibition of RT as the main cause of death of protozoa members of the Trypanosomatidae family [72, 73, 74, 75].

3.5 *COCCINIA GRANDIS* (L.) VOIGT

Coccinia grandis (L.) Voigt popularly known as “Ivy gourd” is a tropical plant belonging to the Cucurbitaceae family. The roots, stems and leaves are used to treat jaundice, bronchitis, rashes, burns, insect bites, fever, indigestion, nausea, eye infections, allergy, syphilis, gonorrhea, etc. (Kirthikar and Basu, 1987; Wasantwisut and Viriyapanich, 2003).

Furthermore, in the study by Das et al., (2015) [76], a crude inhibitor preparation was obtained, which was extracted from fresh leaves of *Coccinia grandis* (L.) Voigt with phosphate buffer. To detect inhibitor activity, the reverse zymography method was used.

To prepare the proteinases, the *L. donovani* cell lysate was prepared according to the protocol by Choudhury et al., (2009) [77].

In the study by Das et al., (2015) [76] it was shown that serine protease inhibitors extracted from *C. grandis* leaves (Cg-Ex) exhibited antiproteolytic activity against *Leishmania* serine protease, with an IC50 of 308,0 ± 2.42 µg/mL, as well as in vitro anti-*Leishmania* activity against *L. donovani*, with an insignificant cytotoxic effect on mammalian macrophages.

In the study by Satheesh and Murugan (2011) [78], the serine protease inhibitor extracted from *C. grandis* demonstrated greater inhibitory activity against trypsin and chymotrypsin, as well as exhibiting antimicrobial and antifungal activity. It was also observed that the serine protease inhibitor of *C. grandis* has demonstrated activity against *Leishmania*, without producing toxic effect against macrophages [79].

Pramanik et al., (2017) [80] also demonstrated that the use of the extract from the leaves of *C. grandis* triggered promastigotes and amastigotes death, through the inhibition of a serine protease, which showed no toxic effect on cells mammals.

In another study, was shown the anti-*Leishmania* activity of the leaf extract of *C. grandis* (L.) Voigt (Cg-Ex), which was supplemented with three serine protease inhibitors (64.8, 55.8 and 15.3 kDa), where the *Leishmania* intracellular serine protease inhibitor (58 kDa, LD-SP) was inhibited by Cg-Ex without showing any toxicity in murine macrophages. The present study revealed that Cg-Ex exerts anti-*Leishmania* activity in a mouse visceral *Leishmania* model, reducing the parasite growth [80]. Therefore, a significant reduction in the parasite load on macrophages by the Cg-Ex treatment indicates a restriction of the parasite development in the host cell.

3.6 *ALOE VERA* (L) BURM F

Aloe vera (L) Burm. f. belongs to the Aloaceae family which includes about 15 genera and 800 species. This species is mainly composed of anthracene derivatives, being the aloins (barbaloin and isobarbaloin) the best known [81, 82]. Studies show that it has anti-inflammatory, healing, antineoplastic and anti-hypoglycemic activity [82, 83].

Regarding the leishmanicidal effect, Dutta et al., (2007) [84], reported that the extract of dried leaves of *A. vera* presented direct effect on axenic promastigotes and amastigotes, and this extract modulated the immune function of murine peritoneal macrophages, causing an increase in reactive oxygen species.

In another study, it was shown that *A. vera* induced programmed death of *L. donovani* promastigotes through a caspase and protease-independent signaling pathway, involving changes in mitochondrial membrane potential and cytochrome C release from hypopolarized mitochondria. resulting in the fragmentation of *L. donovani* DNA.[84].

4 CONCLUSIONS

Given what has been shown, note the importance of further studies involving Leishmania sp proteases as pharmacological targets. Some of these important molecules have already been identified and are characterized as potential candidates for new drugs. Thus, studies with protease inhibitors can lead to the development of new molecules with leishmanicidal activity

When analyzing the studies, it was noted that they use similar protocols for the isolation of protease inhibitor from plant species (affinity chromatography) and proteases from *Leishmania* sp. (cell lysis and protein precipitation and affinity chromatography). Another method used to evaluate the activity of protease inhibitors from plant extracts and also to that of *Leishmania* proteases was reverse zymography, which is the most suitable method for the detection and quantification of the enzymatic activity, as it is capable of detect active and latent forms. This method has a high sensitivity to several classes of enzymes.

However, research involving Leishmania protease inhibitors are still scarce, requiring further studies on the subject, since the understanding of the functioning of these molecules has much to contribute to the development of new therapeutic targets.

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