An \textit{in vitro} study of the antileishmanial and cytotoxic activity of extracts of \textit{Sclerolobium paniculatum} (Fabaceae)

Estudo da atividade \textit{in vitro} antileishmania e citotóxica dos extratos de \textit{Sclerolobium paniculatum} (Fabaceae)

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ABSTRACT

Leishmaniasis is a neglected endemic disease and the pharmacotherapy indicated is expensive, in addition to the unwanted effects generated by the drugs, resulting in low adherence to treatment. Therefore, the search for therapeutic alternatives includes the use of medicinal plants, such as Sclerolobium paniculatum Vogel, which has antioxidant, healing and antifungal properties. Therefore, the aim of this study was to evaluate the in vitro antileishmanial activity of the methanolic and hexanic extracts of S. paniculatum against the promastigote forms of Leishmania (L.) amazonensis and Leishmania (V.) guyanensis and their toxicity in J774 macrophages. As a result, the hexanic extract was considered active against L. guyanensis with an IC$_{50}$ of 40 μg mL$^{-1}$ over 48 hours, with no significant activity for L. amazonensis with an IC$_{50}$ above 100 μg mL$^{-1}$ over 24 and 48 hours. The methanolic extract showed moderate activity against L. guyanensis, with an IC$_{50}$ of 82 μg mL$^{-1}$ over 48 hours and an IC$_{50}$ of 67 μg mL$^{-1}$ over 48 hours against L. amazonensis, making it moderately active. Both extracts did not present cytotoxic profiles at any of the concentrations, being recommended for future research with other species of Leishmania sp. to optimize the treatment of cutaneous leishmaniasis.

Keywords: Cutaneous leishmaniasis; In vitro assay; Therapeutic alternative; Natural products; Taxibranco.

RESUMO

A leishmaniose é uma doença endêmica negligenciada e a farmacoterapia indicada é dispendiosa, além dos efeitos indesejados gerados pelas drogas, resultando assim a baixa adesão ao tratamento. Logo, a busca por alternativas terapêuticas inclui o uso de plantas medicinais, como o Sclerolobium paniculatum Vogel, que possui propriedades antioxidantes, cicatrizantes e antifúngicas. Portanto, este estudo teve como objetivo avaliar a atividade in vitro antileishmaníase dos extratos metanólico e hexânico de S. paniculatum contra as formas promastigotas de Leishmania (L.) amazonensis e Leishmania (V.) guyanensis e a toxicidade em macrófagos de linhagem J774. Como resultado, o extrato hexânico foi considerado ativo contra L. guyanensis com IC$_{50}$ de 40 μg mL$^{-1}$ no período de 48 horas, sem atividade significativa para L. amazonensis com IC$_{50}$ acima de 100 μg mL$^{-1}$ em 24 e 48 horas. O extrato metanólico apresentou atividade moderada contra L. guyanensis, com IC$_{50}$ de 82 μg mL$^{-1}$ no período de 48 horas e com IC$_{50}$ de 67 μg mL$^{-1}$ no período de 48 horas contra L. amazonensis, sendo considerado moderadamente ativo. Ambos os extratos não apresentaram perfis citotóxicos em nenhuma das concentrações, sendo recomendados para pesquisas futuras com outras espécies de Leishmania sp. Para otimização do tratamento da leishmaniose tegumentar.

Palavras-chave: Leishmaniose tegumentar; Ensaio in vitro; Alternativa terapêutica; Produtos naturais; Taxibranco.

INTRODUCTION

Leishmaniasis is an endemic disease caused by protozoa of the genus Leishmania and transmitted through the bite of female sandflies. In 2018, around 92 countries were considered endemic or had reported cases of tegumentary or visceral leishmaniasis, respectively. It is estimated that there are around 1 million new cases of tegumentary leishmaniasis worldwide every year (WHO, 2020).
Leishmaniasis can be found in two clinical forms: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) (ÖZBILGIN et al., 2017; OLIVEIRA et al., 2016). As for the species that cause CL circulating in Amazonas, the following can be highlighted: *L. (L.) amazonensis*, *L. (V.) guyanensis*, *L. (V.) braziliensis* and *L. (V.) naiffi*, with *L. guyanensis* having the highest prevalence (BRASIL, 2017).

The pharmacotherapy recommended by the Ministry of Health consists of the administration of meglumine antimoniate (Glucantime®), pentamidine isethionate (Pentacarinat®) and amphotericin B (Abelcet®) (BRASIL, 2017). The choice of treatment is determined according to epidemiological, laboratory and clinical aspects so that it is possible to identify the species causing the disease and thus assess which drug is the most suitable and efficient for treatment (PELISSARI et al., 2011; VASCONCELOS et al., 2018).

Given these problems with CL pharmacotherapy, there is a need to look for new therapeutic alternatives, one of which is the use of natural products (OLIVEIRA et al., 2016). Medicinal plants have been used to treat diseases since ancient times, according to the World Health Organization (SILVA et al., 2012).

One species with pharmacological potential is *Sclerolobium paniculatum*, which belongs to the Fabaceae family and Caesalpinioideae subfamily (DIAS et al., 1992). This plant is common in Brazil, distributed in the Amazon region, western Bahia, Minas Gerais, Goiás, Mato Grosso and Mato Grosso do Sul (LORENZI, 1992; LORENZI, 1998; CARVALHO, 2003). It can also be known by other popular names in Brazil, such as: taxi-branco, carvoeiro, cachamorra, pau-pombo, among others. In popular medicine, the plant’s bark is used to treat wounds, as well as having antimicrobial, antiphlogistic, antiallergic, contraceptive, diuretic, immunosuppressive and tonic activities (MOLA et al., 1997; FRANCO and BARROS, 2006; CHENG et al., 2008; VIEIRA et al., 2008).

According to Yunes (2001) and Silva et al. (2010), it is widely recognized that the probability of discovering biological activity when investigating a plant that has already been used in folk medicine to treat a specific pathology or symptom is significantly higher than when studying a randomly chosen plant. It is therefore crucial to consider the empirical knowledge of folk medicine.

Research by Silva et al. (2010), identified the presence of alkaloids, flavonoids, tannins and saponins in *S. paniculatum*. Bezerra et al. (1994) also identified in the species: β-sitosterol and stigmasterol (a mixture of steroids), 3β-O-D-Glycopyranosylsitosterol (a
lupane-type triterpene) and Cabreuvin (an isoflavone). It was also possible to observe that the ethanolic extract of *S. paniculatum* showed strong antioxidant potential and after chromatographic fractionation of the extract, the presence of squalene, tocopherols and lupenone was evidenced, predominantly in low polarity fractions (ARANTES, 2011).

According to studies by Stallbaun *et al.* (2017), the wood of *S. paniculatum* promoted high mortality among termites of the genus *Nasutitermes*, observing a high natural resistance of the wood. In addition, other studies have shown that this plant has a high potential for healing lesions and acting against infectious agents (Braga, 1976; Matos, 2007; Lorenzi & Matos, 2008).

Therefore, the aim of this study was to evaluate the antileishmanial activity of the methanolic and hexanolic extracts obtained from *S. paniculatum*, as well as investigating their cytotoxicity in J774 macrophages. With the aim of contributing to the knowledge of medicinal plants with the potential to treat cutaneous leishmaniasis.

**MATERIAL AND METHODS**

This study describes the experimental procedures used in the tests to evaluate the antileishmanial activity of the extracts from the wood residues of *S. paniculatum*. The tests were carried out in partnership with the Thematic Laboratory for the Chemistry of Natural Products - LQPN and the Chagas Disease and Leishmaniasis Laboratory, National Institute for Amazonian Research - INPA.

**Obtaining the material and preparing the extracts**

The extracts were obtained from *S. paniculatum* wood waste at the LQPN/INPA. The waste was crushed in a knife-edge mill to obtain sawdust, which was then macerated in organic solvents in increasing polarity gradients in hexane and then in methanol, both for 7 days each (Cechnel & Yunes, 2001). After this period, the extracts were rotaevaporated and analyzed by comparative thin layer chromatography (CCDC). The crude extracts of *S. paniculatum* were coded for the biological assay as follows: Methanolic Extract (SPM) and Hexanic Extract (SPH), respectively.

**In vitro activities with promastigote forms of Leishmania spp**

The promastigote forms of *L. (V.) guyanensis* (MHOM/BR/1975/M4147) and *L. (L.) amazonensis* (MHOM/BR/2009/IM5584), cryopreserved in the cryobank of the Leishmaniasis and Chagas Disease Laboratory/COSAS/INPA, were used. RPMI 1640
medium supplemented with inactivated fetal bovine serum (iFBS) was used to culture the promastigote forms, and 50 µg mL\(^{-1}\) of antibiotic (gentamicin sulphate - Gentamicin\(^{®}\)) was added and incubated at 25 °C according to Jaffe et al. (1984), for subsequent use in the bioassays.

**Determination of antileishmanial activity and 50% inhibitory concentration (IC\(_{50}\))**

Antileishmanial activity was assessed according to the inhibition of growth and mortality of promastigote forms of *L. amazonensis* and *L. guyanensis*. The extracts were filtered and diluted in the culture medium at concentrations of 125 to 8 µg mL\(^{-1}\). The positive control was Pentacarinat\(^{®}\) (pentamidine isethionate) at concentrations of 10 to 0.6 µg mL\(^{-1}\) and the negative control was DMSO 1% (Dimethylsulfoxide/Merck). The bioassay plate was incubated in an oven at 25°C for 24 and 48 hours. The tests were carried out in triplicate and the average number of live cells was used to calculate the IC\(_{50}\) values according to Fumarola et al. (2004).

**In vitro cytotoxicity tests**

The extracts were tested to determine the effective concentration of 50% of the cell population (EC\(_{50}\)) of uninfected macrophages. J774 macrophages (10\(^5\) cells/mL) were previously grown in a 96-well culture plate containing RPMI 1640 medium supplemented with 10% iFBS (Biosul) for 24 hours. After this period, the old RPMI medium was removed and a new medium containing different concentrations of the extracts was added. The macrophages were incubated in a bacteriological oven at 37 ºC for 48 hours in the presence of the samples (COMANDOLLI-WYREPKOWSKI et al., 2017). After this incubation period, the wells were washed with new medium and then 20 µL of Alamar Blue\(^{®}\) reagent dissolved in PBS was added. The macrophages were incubated for another 5 hours, and then colorimetric readings were taken on a spectrophotometer (Bio-Tek), using wavelengths of 570 nm. The EC\(_{50}\) value was calculated using linear regression.

**Statistical analysis**

The multiplication of parasite cells was determined by sigmoidal curves, using GraphPad Prism 8.0 software, analyzing the respective 95% confidence intervals and linear coefficients.
RESULTS AND DISCUSSION

Plants have been used to treat a range of ailments since the dawn of humanity, with the first reports dating back to 3,000 BC in China (LOPES et al., 2018). According to the World Health Organization, around 85% of the world's population uses medicinal plants for health care and 80% of the population in developing countries uses traditional approaches as part of their basic health care (SOUZA et al., 2016). In 1996, the 10th National Health Conference debated the implementation of alternative therapies and popular practices in the Unified Health System (SUS), especially regarding phytotherapy and homeopathy, emphasizing once again the importance of observing popular practice in health research and treatment (DE-FREITAS et al., 2014).

In recent years, scientific and technological advances have led to the discovery of many new molecules and their mechanisms of action through the study of plants, proving their medicinal activity, a factor that should be explored (FÜRST & ZÜNDORF, 2015).

As an example, the species *Myroxylon peruiferum* L., belonging to the Fabaceae family, has been reported for use in scientific and folk medicine. It is popularly used against wounds and ulcers, headaches, torticollis, and tuberculosis. Its most important compound is cabreuvin, which has proven activity against *Helicobacter pylori*, and its ethanolic extract has shown antimalarial activity *in vitro* (MUÑOZ et al., 2000; ANDRADE et al., 2016; PEREIRA et al., 2019).

The Fabaceae family is being investigated in studies with antileishmanial and antifungal activities, as in the studies by Santana et al. (2015), with *Enterolobium ellipticum* Benth, *Sclerolobium aureum* (Tul.) Baill. and *Vatairea macrocarpa* (Benth.), and Toledo et al. (2010) with *S. aureum* which showed activity against *Candida* species and *S. aureum* (Tul.) Baill. which demonstrated antimalarial activity (MUÑOZ, et al., 2000). Rodrigues and Cavalcante (2022) carried out studies to assess the larvicidal potential of the hydroalcoholic extract of *S. paniculatum* for 72 hours at various concentrations against *Aedes aegypti*, obtaining positive results with its highest concentrations, reaching 100% mortality. In view of these results, this same plant species was used in this study to evaluate its activity against *Leishmania* sp., from the methanolic and hexanic extracts, which were subjected to *in vitro* biological tests against the promastigote forms of *L. amazonensis* and *L. guyanensis*, within 24 and 48 hours.
In the test carried out against the *L. amazonensis* species, the hexanic extract (HPS) of *S. paniculatum* did not obtain significant results in terms of inhibiting parasite activity during the 48-hour test when compared to the action of Pentacarinat®, as can be seen in graph A of Figure 1. In relation to the results obtained with the methanolic extract seen in graph B of Figure 1, there was a small difference in the number of promastigotes between the two time periods evaluated, however there was no significant parasite inhibition.

**Figure 1** - Activity of the hexanic (A) and methanolic (B) extracts at 24 and 48 hours against *L. amazonensis*.

Regarding the *in vitro* test carried out against the *L. guyanensis* species, the SPH extract showed effectiveness against this species, as can be seen in graph A of Figure 2, in which during the 48-hour test it showed notable parasite inhibition. On the other hand, although the SPM extract had inhibitory activity in the first 24 hours, there was an increase in the concentration of promastigotes in the following hours, resulting in the extract losing its effectiveness, as can be seen in graph B of Figure 2.

**Figure 2** - Activity of methanolic (A) and hexanolic (B) extracts at 24 and 48 hours against *L. guyanensis*.

To determine the antileishmanial activity of the samples, the 50% inhibitory concentration is calculated, which can be measured using the work of Osorio *et al.* (2007),
classifying the samples as: highly active (IC$_{50}$ < 10 μg mL$^{-1}$), active (IC$_{50}$ between 10-50 μg mL$^{-1}$), moderately active (IC$_{50}$ between 50-100 μg mL$^{-1}$) and not active (IC$_{50}$ > 100 μg mL$^{-1}$).

In the test carried out against the *L. amazonensis* species, the hexanic extract (SPH) of *S. paniculatum* showed IC$_{50}$ values above 100 μg mL$^{-1}$ in the 24 and 48 hour periods and was not characterized as active in the periods analysed (Table 1). On the other hand, the methanolic extract (SPM) showed a IC$_{50}$ greater than 100 μg mL$^{-1}$ in the 24 hour period, and in the 48 hour period it showed a IC$_{50}$ of 67 μg mL$^{-1}$, making it a moderately active extract.

**Table 1** – IC$_{50}$ values in an *in vitro* test against *L. amazonensis* promastigotes of the hexanic (SPH) and methanolic (SPM) extracts of *S. paniculatum*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>L. amazonensis</th>
<th></th>
<th>L. guyanensis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
<td>48h</td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>SPH</td>
<td>&gt;100 ± 3.6</td>
<td>&gt;100 ± 2.6</td>
<td>&gt;100 ± 2.5</td>
<td>40 ± 2.5</td>
</tr>
<tr>
<td>SPM</td>
<td>&gt;100 ± 4.2</td>
<td>67 ± 3.0</td>
<td>32 ± 1.7</td>
<td>82 ± 2.5</td>
</tr>
<tr>
<td>Pentacarinat®</td>
<td>&lt;0.6 ± 0.1</td>
<td>&lt;0.6 ± 0.1</td>
<td>&lt;0.6 ± 0.1</td>
<td>&lt;0.6 ± 0.01</td>
</tr>
</tbody>
</table>

In the test against the species *L. guyanensis*, the extracts showed more significant results, with the hexanic extract proving to be more active in the 48-hour period, with an IC$_{50}$ of 40 μg mL$^{-1}$. The methanolic extract showed an IC$_{50}$ of 32 μg mL$^{-1}$ in the 24-hour period and 82 μg mL$^{-1}$ in the 48-hour period, varying between active and moderately active in these periods (Table 1).

In this way, we can consider that, for *L. amazonensis*, the methanolic extract was more effective than the hexanic extract and was considered moderately active within 48 hours. And for the test against *L. guyanensis*, the hexanolic extract was more active over the same period.

Regarding the cell cytotoxicity test with J774 macrophages, no cytotoxic profiles were observed at any of the concentrations of both *S. paniculatum* extracts (125 μg mL$^{-1}$ to 8 μg mL$^{-1}$), as can be seen in Figure 3, showing high cell viability. Only pentamidine isethionate - Pentacarinat®, showed cytotoxic potential as expected, because despite being considered one of the standard drugs for the treatment of cutaneous leishmaniasis, it is a drug with many adverse reactions, such as hepatotoxicity and nephrotoxicity (BRASIL, 2017).
The study by Toledo et al. (2011) may corroborate the results obtained in this study, as they also found no cytotoxic activity against human erythrocytes. According to the literature, Sclerolobium aurem is used in communities for the treatment of fungal infections and in infusion form as a hepatoprotector, which suggests that the species is also non-toxic in moderate concentrations, which reinforces the result obtained in the present study (SANTANA et al., 2015).

Figure 3 - Cytotoxic activity of the samples tested on J774 macrophages incubated at 37ºC for 24 and 48 hours, assessed by cell viability. A - SPH (hexane extract); B - SPM (methanolic extract); C - GLUC (meglumine antimoniate - Glucantime®) and D - PTM (pentamidine isethionate - Pentacarinat®). The negative control was 1% DMSO (dimethyl sulfoxide).

As for the phytochemical classes found in this plant, Silva et al. (2010) identified the presence of flavonoids, tannins, terpenes and saponins in S. paniculatum and Arantes (2011) identified the presence of flavonoids in hydroalcoholic extract and in hexanic extract identified the presence of squalene, α-tocopherol, lupenone and flavonoids.

According to Feng et al. (2018), lupenone is a lupane-type triterpenoid, which has anti-inflammatory, anticancer and antiviral effects, and is also used against Chagas disease, without showing great toxicity in the experiments. Feng et al. (2020) also states that lupenone has anti-inflammatory activity and evaluates its effects on acute and subacute diabetic pancreatic inflammation. These compounds acting synergistically may have contributed to the anti-parasitic results found in this study.
Squalene, for example, one of the substances already found in this plant, is a triterpene used in the pharmaceutical industry to improve the oral absorption of therapeutic molecules as an excipient in vaccines and medicines. In addition, according to Reddy and Couvreur (2009), it has been shown to stop or prevent tumor growth in experimental tumors. Squalene accumulates in high concentrations in the skin, acting to quench free radicals in the skin and, according to the author, has a bright future in disease therapy.

Among the extracts, the hexanic showed the greatest activity, probably due to the presence of substances found in it that confer anti-inflammatory and antioxidant action. However, it is worth pointing out that it is extremely important to carry out further studies to assess the concentration and isolation of the substances to evaluate their anti-leishmanial potential in a monotherapeutic way and really understand their mechanism of action against the parasite.

Based on the promising results with the extracts obtained from *S. paniculatum*, especially the hexane, and because they have not shown a cytotoxic profile, it is highly recommended that further research be carried out on the *L. guyanensis* species. Such efforts could potentially improve the recommended pharmacotherapy and lead to the development of a more effective treatment that is less harmful to health, thus promoting greater patient compliance and reducing the financial burden on the health system.

**CONCLUSION**

The hexanic extract showed the greatest activity against *L. guyanensis* with an IC$_{50}$ of 40 µg mL$^{-1}$ within 48 hours and was considered active. The methanolic extract showed moderate activity against *L. amazonensis* and *L. guyanensis* with IC$_{50}$ values of 67 and 82 µg mL$^{-1}$, respectively over 48 hours. Another very positive point was that both extracts did not show cytotoxic profiles. Thus, considering the results obtained, it is essential to conduct more detailed investigations aimed at identifying the chemical compounds contained in these extracts. This, in turn, will allow subsequent evaluations to be carried out using other *in vitro* and *in vivo* tests.
REFERENCES


