Evaluation of activity of *Matricaria chamomilla* against *Leishmania amazonensis*

**Avaliação da atividade de Matricaria chamomilla contra Leishmania amazonensis**

**Evaluación de la actividad de Matricaria chamomilla contra Leishmania amazonensis**

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ABSTRACT

Leishmaniasis is a tropical disease caused by different species of Leishmania. The available treatments have limitations linked to high toxicity and low efficacy. Thus, the search for new antileishmanial compounds is needed. Since Matricaria chamomilla L. have several activities described in literature, the aim of this search was to obtain crude extract and fractions of M. chamomilla and to evaluate the activity against the evolutionary forms of Leishmania amazonensis. Floral chapters of M. chamomilla were preserved in 95% ethanol for 12 days and lyophilized to obtain the crude extract (CE). Then, with the aid of a separating funnel and organic solvents, hexane (FHE), ethyl acetate (FEA) and hydromethanolic (FHM) fractions were obtained. The CE and the fractions were tested in vitro against the promastigote and intracellular amastigotes forms and the macrophage cell line J774A.1. The results were plotted on graphs and the IC\textsubscript{50} was determined in relation to the control. In view of the evolutionary forms of L. amazonensis, FHE presented the best results, with the IC\textsubscript{50} for promastigotes of 14.2 µg/mL, with a selectivity index (IS) of 8.15; Against amastigotes, FHE showed an IC\textsubscript{50} of 53.84 and IS of 2.13. Further studies should be conducted to elucidate FHE's antileishmanial actions.

Keywords: chamomilla, Leishmaniasis, antileishmanial activity.

RESUMO

A leishmaniose é uma doença tropical causada por diferentes espécies de Leishmania. Os tratamentos disponíveis têm limitações ligadas à elevada toxicidade e à baixa eficácia. Assim, é preciso procurar novos compostos antileishmanianos. Como a Matricaria chamomilla L. possui várias atividades descritas na literatura, o objetivo desta pesquisa foi obter extrato bruto e frações de M. chamomilla e avaliar a atividade contra as formas evolutivas da Leishmania amazonensis. Capítulos florais de M. chamomilla foram preservados em etanol a 95% por 12 dias e liofilizados para obter o extrato bruto (CE). Em seguida, com o auxílio de uma ampola de decantação e solventes orgânicos, foram obtidas frações de hexano (FHE), acetato de etila (FEA) e hidrometanólico (FHM). O CE e as frações foram testados in vitro contra as formas promastigota e amastigota
intracelular e contra a linha celular dos macrófagos J774A.1. Os resultados foram
plotados em gráficos e o IC50 foi determinado em relação ao controle. Em vista
das formas evolutivas de L. amazonensis, a FHE apresentou os melhores
resultados, com o IC50 para promastigotes de 14,2 µg/mL, com índice de
seletividade (IS) de 8,15; Contra amastigotas, a FHE apresentou IC50 de 53,84
e IS de 2,13. Estudos adicionais devem ser realizados para elucidar as ações
antileishmanianas da FHE.

**Palavras-chave:** camomila, *Leishmaniose*, atividade antileishmanial.

**RESUMEN**
La leishmaniasis es una enfermedad tropical causada por diferentes especies de
Leishmania. Los tratamientos disponibles tienen limitaciones relacionadas con la
alta toxicidad y la baja eficacia. Por lo tanto, se necesita la búsqueda de nuevos
compuestos antileishmaniales. Dado que Matricaria chamomilla L. tiene varias
actividades descritas en la literatura, el objetivo de esta búsqueda fue obtener
extracto crudo y fracciones de M. chamomilla y evaluar la actividad frente a las
formas evolutivas de Leishmania amazonensis. Los capítulos florales de M.
chamomilla se conservaron en etanol al 95% durante 12 días y se liofilizaron
para obtener el extracto crudo (CE). Luego, con la ayuda de un embudo de
separación y disolventes orgánicos, se obtuvieron fracciones de hexano (FHE),
acetato de etilo (FEA) e hidrometanólico (FHM). El CE y las fracciones se
probaron in vitro contra las formas de promastigote y amastigotes intracelulares
y la línea celular de macrófagos J774A.1. Los resultados se trazaron en gráficas
y se determinó la CI50 en relación con el control. En vista de las formas
evolutivas de L. amazonensis, la HE presentó los mejores resultados, con la IC50
para promastigotes de 14,2 µg/mL, con un índice de selectividad (IS) de 8,15;
frente a los amastigotes, la FHE presentó una IC50 de 53,84 e IS de 2,13.
Deberían realizarse más estudios para dilucidar las acciones antileishmaniales
de la FHE.

**Palabras clave:** chamomilla, *Leishmaniasis*, actividad antileishmanial.

**1 INTRODUCTION**
Leishmaniasis are a group of diseases caused by species of flagellated
protozoa of the genus *Leishmania* belonging of the Trypanosomatidae family.
They are responsible for 30,000 deaths per year, worldwide. These protozoa are
obligate intracellular parasites and there are more than twenty species
documented which can infect human race (ORYAN et al., 2018; TAGHIPOUR et
al., 2021). Dogs, rodents, edentates and echinus can act as reservoirs of
*Leishmania*, the transmission occurs exclusively through mosquitoes' bites of
The parasite has a heteroxenous life cycle, alternating between invertebrates and vertebrates' hosts. There are two main life stages, the promastigote - oval, elongated and flagellated shape, found in the mosquito's digestive tract; and the amastigote - rounded with a non-exteriorized flagellum which is found in the vertebrate host (SERENO et al., 2019).

There are three possible manifestations of the disease: cutaneous, mucosal and visceral. The cutaneous form consists in multiple papules, crusted nodules, ulcerative plaques or nodular lesions. The mucosal form, in opposition, presents with intensively destructive lesions in mucous membranes. Meanwhile, the visceral or kala-zar form is the most intense and severe type, it is associated with intense, prolonged fever, fatigue, hepatosplenomegaly and death (BURZA et al., 2018; AKBARI et al., 2017).

The treatment is chosen by region, numerous possible agents and clinical manifestations. Few options are: surgical excision, symptomatic treatment, cryotherapy, thermotherapy, laser and ointments for topic treatment (CARDONA-ARIAS et al., 2015; JAFFARY et al., 2016; ORYAN & ALEMZADEH, 2016; SUNDAR & CHAKRAVARTY, 2015). Concomitantly, specific drugs are used for chemotherapy, being the classical treatment composed of pentavalent antimonials (Meglumine antimoniate - Glucantime® - and sodium Estibogluconate - Pentostam®-); pentamidine and Amphotericin B can be used as well as a second choice (ANDRADE et al., 2016; BAHRAMI et al., 2016; BURZA et al., 2018). Those are usually effective choices, though they can be limited by high toxicity, painfulness, long term treatment, high costs and the necessity of intravenous administration (GARCIA et al., 2013; SERENO et al., 2019; BURZA et al., 2018).

Facing all the obstacles imposed by the disposable drugs, as well as the growing increase in resistance to the existing drugs (BURZA et al., 2018), there is an urgent necessity for studies that aims the discovery of new compounds with an anti-\textit{Leishmania} activity. One possible alternative for studies is the
phytotherapeutic compounds; in this context, it can be highlighted the plant *Matricaria chamomilla* L.  

*Matricaria* is a genus of flowers from the Asteraceae family, it is a dicotyledon found in temperate regions of the world (SINGH et al., 2011). It is also known scientifically as *Chamomilla courrantiana*, *Matricaria chamomilla*, *Matricaria chamomilla* var. *recutita*, *Matricaria courrantiana*, *Matricaria recutita* and *Chamomilla recutita* (ZEMESTANI et al., 2016). There are few traditions in the use of *M. chamomilla* as a medicinal plant, since hundreds of years ago, being present in Egyptian, middle aged, old Persian and also in Mediterranean medicine (SHARIFI-RAD et al., 2018). Currently, *M. chamomilla* is used in great quantities in homeopathic formulations and in Unani medical practice, specially its flowers, stem and essential oil (MEHMOOD et al., 2015).

The flower buds of *M. chamomilla* should be used dry and stored in a dark place, to prevent it from oxidation (MATOS, 2007). In traditional medicine, there are descriptions of its use as spasmolytic, anti-inflammatory, wound healing and regenerating. It is usually used as a tea, orally, to abdominal discomforts, dyspepsia, gastrointestinal ulcers, common cold, bronchitis, gastrointestinal spasms, epilepsy, hypertension, neuralgia, odontalgia, dysmenorrhea, eczema and impetigo, diarrhea and for a sedative effect. It is also cited as a hemorrhoid treatment, for aphthous stomatitis, scars, blisters, sun burning, rashes and conjunctivitis (TOLOUEE et al., 2010; MEHMOOD et al., 2015).

There are a few case reports in literature of allergic and autoimmune disorders, such as dermatitis or rashes, after the topical use of *M. chamomilla*. In spite of that, camomile was listed as “Generally Recognized as Safe” (GRAS) by FDA, since it does not present any potentially toxic compound, therefore, it doesn’t present acute toxicity to humans or animals (JAMALIAN et al., 2012; TOLOUEE et al., 2010; TADRENT et al., 2016).

In the scientific literature, it was found several properties and activities of *M. chamomilla* as anti-inflammatory (CARNAT et al., 2003; PEÑA, 2006), gastroprotective (MOURA ROCHA et al., 2010; MENALE et al., 2021), anti-diarrheal and antioxidant (SEBAI et al., 2014), neuroprotector (RANPARIYA et
al., 2011), hepatoprotective (BABENKO and SHAKHOVA, 2006), antiallergenic (CHANDRASHEKHAR et al., 2011), antimicrobial - even against antibiotic-resistant bacteria - (SILVA et al., 2012; SADAT et al., 2021), cardioprotector effect (AWAAD et al., 2018), anti-diabetic (RAFRAF et al., 2015), anti-mutagenic and anti-cancer (NIKSERESHT et al., 2017).

After research that evidenced the relevance of Matricaria chamomilla in several properties, including dermatological and antimicrobial characteristics, this study aimed to evaluate its in vitro activity against Leishmania amazonensis. In this context, crude extract and fractions of M. chamomilla were prepared and evaluated antileishmanial properties and cytotoxicity over macrophages.

**2 MATERIAL AND METHODS**

*Matricaria chamomilla* flower buds were purchased from Ervanário (Maringá, PR, Brazil). Dimethyl sulfoxide (DMSO), hexane, folic acid, hemin, Warren’s medium, thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brain heart infusion (BHI) was acquired from Beckon Dickinson (Sparks, MD, USA). Fetal bovine serum (FBS), RPMI 1640, Dulbecco’s Modified Eagle’s medium (DMEM) and Giemsa were obtained from Invitrogen (Grand Island, NY, USA). All of the reagents were of analytical grade.

**2.1 OBTENTION OF THE CRUDE EXTRACT AND FRACTIONS OF M. Chamomilla**

The crude extract was obtained from dry and stabilized flower buds. The vegetative portion was macerated with the liquid extractor, 96% ethanol, in a ratio of 15.0 g of flower buds to 175 mL of solvent, totalizing 1,000 g of flower buds. The plant material was kept bottled with the solvent for twelve days, protected from direct light. Then, the solution was filtered, followed by a rotoevaporation of the solvent, and lyophilization. It was then packed and kept in an appropriate place in the absence of light.

The crude extract was successfully obtained and resulted in 28.6 g; part of it was stored for posterior experiments and another part was used for the
partition with different solvents. The fractions were obtained from the crude extract, which was previously prepared, by the liquid-liquid extraction method. For that, 25.0 g of crude extract was first diluted in a methanol-water solution (1:1), then it was packed within a separation funnel and submitted to a successive partitioning with increasing polarity solvents: hexane (5 L) and ethyl acetate (3 L). The residual fraction was named hydromethanolic fraction. The solvents were rotoevaporated at 40 °C, followed by lyophilization. The samples obtained were named as crude extract, hexane fraction, ethyl acetate fraction and hydromethanolic fraction.

2.2 PARASITES AND MACROPHAGES J774A1 CULTURE

*Leishmania amazonensis* promastigotes (WHOM/BR/75/JOSEFA strain) - isolated from a human diffuse cutaneous leishmaniasis case - were cultivated in Warren medium (Heart and Brain infusion “Difco®”, hemin and folic acid), pH 7.0, supplemented with 10% of fetal bovine serum (FBS - Gibco Invitrogen, Gaithersburg, MD, EUA), and were incubated at 25 °C. J774A1 macrophages were maintained in RPMI 1640 medium supplemented with 10% FBS, at 37 °C with 5% CO₂.

2.3 EVALUATION OF ACTIVITY OF CRUDE EXTRACT AND FRACTIONS OF *M. CHAMOMILLA* AGAINST *L. AMAZONENSIS* PROMASTIGOTES

For assay to evaluate the promastigote forms of *L. amazonensis*, the protozoa were plated in 96-well sterile plates, with a concentration of 1 x 10⁶ cells/mL, containing Warren medium and supplemented with 10% FBS. The protozoa were treated with crescent concentrations (1 – 1000 µg/mL) of fractions, crude extract of *M. chamomilla* or growing medium alone (used as the control). The plates were incubated for 72 h at 25 °C. After, a solution was prepared with the content of the wells combined with 3% formalin and the count of viable promastigotes took place using a Neubauer chamber. The results were expressed as the inhibition percentage compared to the control as IC₅₀.
2.4 EVALUATION OF ACTIVITY OF CRUDE EXTRACT AND FRACTIONS OF M. CHAMOMILLA AGAINST L. AMAZONENSIS INTRACELLULAR AMASTIGOTES

Round coverslips were placed in 24-well plates, on them were dispensed a suspension of cells containing macrophages (5 x 10^5 cells/mL) and promastigotes (5 x 10^6 parasites/mL), 6 days after cultivated, in RPMI 1640 medium and 10% FBS, incubated for 24 h at 34 °C and 5% CO_2. Then, the treatment was performed with different concentrations (1 – 1000 µg/mL) of crude extract, its fractions and a sample with medium (control); the plates were incubated for 48 h under the same conditions. Next, the fixation was made with methanol for 20 min, stained with Giemsa 5% for 20 min and placed over a slide with Permount. The counting was performed with an optical microscope and the survival index was calculated (by multiplying the percentage of infected macrophages by the average of parasites per macrophages). The results were expressed as the inhibition percentage compared to the control as IC$_{50}$ and the IC$_{90}$.

2.5 CYTOTOXICITY EVALUATION OF THE CRUDE EXTRACT AND FRACTIONS OF M. CHAMOMILLA AGAINST MACROPHAGES J774A1

In 96-well plates, macrophages (5 x 10^5 cél/mL) were cultivated in RPMI 1640 medium with 10% FBS, at 37 ºC, 5% CO$_2$, for 24 h. Next, the M. chamomilla extract and fractions were added in different concentrations (1 – 1000 µg/mL), and incubated for 48 h in the same conditions. Then, the cells were washed with PBS twice and cell viability was assessed by MTT assay, based on the cellular conversion of tetrazolium salt into formazan in the presence of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (GRELA et al., 2018).

The MTT (2 mg/mL) was added to each well, followed by 4 h incubation at 37 °C, 5% CO$_2$. After, MTT solution was removed and DMSO was added in the wells, in order to solubilize the crystals. The absorbance of each well was read on a microplate reader (Power Wave XS - Bio-Tek) at 570 nm. The percentage of viable cells were calculated compared to the control and the cytotoxic
concentration for 50% of cells (CC\textsubscript{50}) was determined in relation to the control.

### 3 RESULTS

The crude extract was successfully produced and yielded an amount of 28.16 g. Twenty-five grams of this amount was used to produce the three fractions. The quantity obtained of each fraction is described in Table 1. Hexanic fraction presented the best performance (50.6%; 12.66 g).

Table 1. Yield of the hexanic, ethyl acetate and hydromethanolic fractions from the crude extract of M. chamomilla

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yield (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanic</td>
<td>12.66</td>
<td>50.6</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>5.7</td>
<td>21.0</td>
</tr>
<tr>
<td>Hydromethanolic</td>
<td>6.7</td>
<td>26.0</td>
</tr>
</tbody>
</table>

Source: Authors

Furthermore, the experiments against L. amazonensis promastigotes yielded high quality data to each fraction and to the crude extract. The inhibitory concentration values to 50% (IC\textsubscript{50}) against the promastigote forms were calculated and expressed as average ± standard deviation (µg/mL). The IC\textsubscript{50} values are listed in Table 2. It is worth noting that the hexanic fraction presented the lowest and the best outcome, with an IC\textsubscript{50} of 14.2 ± 1.17 µg/mL.

Further, assays were performed to elucidate cytotoxicity of the extract and its fractions against macrophages in order to find the cytotoxic concentrations values of 50% (CC\textsubscript{50}). The results obtained are included in Table 2. The less cytotoxic fraction was the hydro methanolic, followed by the hexanic and then, the ethyl acetate fraction.

The selectivity index (SI) of each compound was calculated as well. This index makes reference to the ratio between the J774A.1 cells CC\textsubscript{50} and the protozoa IC\textsubscript{50}. These data are presented in Table 2 and it is possible to conclude that the best SI was to the hexane fraction.

Lastly, an essay was performed to test the inhibition of each compound against L. amazonensis amastigotes. The data were analyzed also by means of the IC\textsubscript{50} and the crude extract, ethyl acetate and hydro methanolic fractions.
presented values above 100 µg/mL. Interestingly, the hexanic fraction was the only active against this parasitic form, exhibiting an IC\textsubscript{50} of 75.44 ± 18.75 µg/mL.

Table 2. Inhibitory and cytotoxic values to 50% of the cells for the crude extract and fractions against promastigotes and amastigotes of \textit{L. amazonensis} and J774.A1 macrophages

<table>
<thead>
<tr>
<th></th>
<th>Macrophages CC\textsubscript{50} (µg/mL)</th>
<th>Promastigotes CI\textsubscript{50} (µg/mL)</th>
<th>SI promastigote</th>
<th>Amastigotes CI\textsubscript{50} (µg/mL)</th>
<th>SI amastigote</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>74.4 ± 3.8</td>
<td>69.0 ± 4.0</td>
<td>1.1</td>
<td>&gt;100</td>
<td>-</td>
</tr>
<tr>
<td>Hexanic</td>
<td>115.8 ± 10.9</td>
<td>14.2 ± 1.2</td>
<td>8.2</td>
<td>75.4 ± 18.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>84.0 ± 2.8</td>
<td>30.4 ± 4.7</td>
<td>3.1</td>
<td>&gt;100</td>
<td>-</td>
</tr>
<tr>
<td>Hydro methanolic</td>
<td>734.2 ± 62.1</td>
<td>725.8 ± 98.9</td>
<td>1.0</td>
<td>&gt;100</td>
<td>-</td>
</tr>
</tbody>
</table>

Source: Authors

4 DISCUSSION

Our research group have already demonstrated that the plants of Astaraceae family have antiparasitic properties, as the case of the crude extract of \textit{Tanacetum parthenium}, which has activity against both main parasitic forms of \textit{L. amazonensis} (Rabito et al., 2014), and \textit{Anthemis tinctoria}, which exhibited activity against \textit{Trypanosoma cruzi} (Bittencourt et al., 2011). Moreover, in a recent study, it was described that the essential oil of \textit{M. chamomilla} presented activity against promastigote and amastigote forms of \textit{L. amazonensis} (Karam et al., 2020). Thus, both crude extract and fractions obtained from the flower buds of \textit{M. chamomilla} were the object of interest in this study, which were successfully prepared. The decision to obtain the crude extract from the flower buds was based on the fact that higher concentration of volatile compounds and sesquiterpenes are mostly secreted by glandular ducts localized on the flowers (SHARIFI-RAD et al., 2018).

The crude extract and ethyl acetate fraction handed over activity against promastigotes, however, neither of them presented activity against amastigotes in the tested concentrations. The amastigote is the evolutive form of clinical interest, since it is the prevalent form that can be founded in infected patients (SPOTIN et al., 2015). In opposition, the hydro methanolic fraction proved to be the less toxic fraction and provided activity against promastigotes, however, with an extremely high dosage, thus did not present an interesting selectivity.
Regarding the results with amastigotes, the hydro methanolic fraction also did not show any activity against this parasitic form. For both promastigotes and amastigotes tests, the hexane fraction showed the best results, since it presented SI of 8.15 and 1.53 for the promastigotes and amastigotes, respectively.

The concentrations of compounds present in composition of *M. chamomilla* vary according to the plant region of origin, genetic and other environmental factors (EL MIHYAOUI et al., 2022). It has already been well described that the main components present in the *M. chamomilla* are volatile terpenoids (α-bisabolol [5-70%], bisabolol oxide A [5-60%] and B [5-60%], β-trans-farnesene [7-45%] and chamazulene [1-35%]), sesquiterpene lactones (matricin) and also phenolic compounds (flavonoids, coumarins and phenolic acids) (SHARIFI-RAD et al., 2018; KAZEMI, 2014; EL MIHYAOUI et al., 2022.). Volatile compounds present in the crude extract and essential oil of *M. chamomilla* and several other plants, such as mono- and sesquiterpenes, have been proofed to exert several activities in the human organism (ADORJAN and BUCHBAUER, 2010), such as antinociceptive, gastroprotective (MOURA ROCHA et al., 2010), antiinflamatory (ASADI et al., 2020), antiviral (Koch et al., 2008), and insect repellent (HÖFERL et al., 2020). This data corroborates with our theory that those could be the substances that influenced the antileishmanial effects in this study.

Our results showed that the hexane fraction presented the best SI, and this can be explained based on the fact that hexane is a non-polar solvent, frequently used for oil extraction in several situations. Furthermore, it is a hydrocarbon insoluble in water, with a great affinity for non-polar compounds, such as sesquiterpenes (JØRGENSEN and COHR, 1981). In addition, Bouazzi et al. (2020) were able to proof such affinity characterizing the composition of *Onopordum arenarium* hexane extract: 39.6% of its content was identificated as triterpenoids, 27.3% hydrocarbons, 5.5% sterols, and the remaining was characterized as fatty acids, alcohols and others. Thereby, it is likely that the compounds with antileishmanial activity in the flower buds of *M. chamomilla* were sesquiterpenes and other terpenoids; which probably are present in hexane
fraction in higher concentrations, being the fraction that demonstrated the greatest antileishmanial potential.

The results obtained in this study provide preliminary information that show that the hexane fraction can be auspicious regard an antileishmanial activity, nevertheless, more studies should be conducted to better elucidate this fraction’s safety, as well as its mechanism of action and characterization regarding its major compounds.

5 CONCLUSION

The crude extract and its fractions were successfully obtained from the flower buds of *M. chamomilla*. It is possible to conclude that, between the crude extract and the fractions, the hexanic fraction was the one with the best antileishmanial activity, since it was the fraction with the best IC$_{50}$ against parasites and the higher selectivity index. Therefore, the results demonstrated that the hexanic fraction is a promising therapeutic agent against leishmaniasis that should be further investigated, for a complete characterization of its activities in face of the evolutionary forms of *L. amazonensis*.

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