

Antileishmanial activity of the methanol extract of *Cedrela odorata*: growth inhibition and mortality of *Leishmania* spp**Atividade antileishmaniana do extrato metanólico de *Cedrela odorata*: inibição do crescimento e mortalidade de *Leishmania* spp**

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Abstract

Across the planet, woody species of the Meliaceae family have suffered a significant reduction over the centuries due to factors such as deforestation, fires and logging, and are currently at risk of extinction. This scenario has made phytochemical studies of its wood scarcer, even though knowledge of the chemical profile and biological potential of its compounds are essential in order to increase its scientific value. Thus, wood residues provide a new possibility to continue these studies. For this study, wood residues of *Cedrela odorata* L. (pink cedar) were used from window frames that were obtained from after the demolition of a forty-five-year-old building at the Instituto Nacional de Pesquisas da Amazônia (INPA). The chromatographic fractionation process of the methanol extract resulted in the isolation of two limonoids: gedunin and mexicanolid. However, only the mexicanolid and the methanolic extract were tested against the species *Leishmania amazonensis* and *guyanensis*, though both compounds demonstrated antileishmanial activity, with the IC₅₀ being between 50-100 µg mL⁻¹ for the promastigote form of *L. amazonensis* and between 10-50 µg mL⁻¹ against the species *L. guyanensis* (*in vitro*).

Keywords: Waste wood, *Cedrela*, Limonoid, *Leishmania*.

Resumo

Em todo o planeta, espécies lenhosas da família Meliaceae tiveram uma redução significativa ao longo de séculos devido à fatores como desmatamentos, incêndios e exploração e estão atualmente em risco de extinção. Este cenário tornou mais escasso os estudos fitoquímicos com a sua madeira, mesmo que o conhecimento do perfil químico e do potencial biológico dos seus compostos sejam fundamentais para ampliar seu valor científico. Dessa forma, os resíduos madeireiros proporcionam uma nova possibilidade para dar continuidade a esses estudos. Para esta pesquisa, utilizou-se resíduos madeireiros de *Cedrela odorata* L (cedro-rosa) de esquadrias de janelas, as quais foram obtidas a partir da demolição de um prédio do Instituto Nacional de Pesquisas da Amazônia o qual tem quarenta e cinco anos de idade. O processo de fracionamento cromatográfico do extrato metanólico; resultou no isolamento de dois limonóides: gedunina e mexicanolida. Porém, apenas o mexicanolida e o extrato metanólico foram testados contra as espécies *Leishmania amazonensis* e *guyanensis*, e ambos os compostos demonstraram possuir atividade antileishmaniana, sendo o IC₅₀ entre 50-100 µg mL⁻¹ contra a forma promastigota de *L. amazonensis* e entre 10-50 µg mL⁻¹ contra a espécie *L. guyanensis* (*in vitro*).

Palavras-chave: Resíduos madeireiros, *Cedrela*, Limonóide, *Leishmania*.

Introduction

For a long time, some species of the Meliaceae family have suffered great exploitation, mainly for use in civil construction, and this has led to the depletion of many of their natural populations in various regions of the planet. In addition, these species were often employed in the manufacture of fine furniture¹⁻⁵. Since then, in order to avoid the deforestation of native forests, phytochemical studies carried out on the wood of these species have had to find other means of obtaining their secondary metabolites. In this sense, the use of wood residues has proved to be a viable alternative for these studies. From the wood residues of *C. odorata*, it was possible to carry out a preliminary evaluation of the methanolic extract, which led to the isolation of two limonoids, for which the methanolic extract and one of these limonoids was tested against the promastigote form of the species *Leishmania amazonensis* and *Leishmania guyanensis*.

Tegumentary leishmaniasis (TL) is one of the most relevant neglected tropical diseases in the world due to the large number of people infected and the complexity of the levels of clinical evolution of patients⁶. This disease has been reported in 98 countries and about 2 to 2.5 million new cases occur per year. Its transmission occurs in a vectorial form via the female phlebotomines of the genus *Lutzomyia* when infected with the protozoan of the genus *Leishmania*⁶⁻¹⁰. Among the species causing TL circulating in the Amazon, we can highlight *L. amazonensis*, *L. guyanensis*, *L. braziliensis* and *L. naiffi*; though *L. guyanensis* is the most prevalent⁶.

In the treatment of TL, pentavalent antimonials were the first drugs to be adopted as therapy, followed by the use of amphotericin B, pentamidine, pentoxifylline and miltefosine, which are still currently included in the therapeutic protocol indicated by the Brazilian Ministry of Health⁶. However, the therapeutic indication is determined according to epidemiological, laboratory and clinical aspects in order to identify which species is the cause of the disease and, thus, evaluate which medication is the most appropriate and efficient for treatment^{11, 12}. However, these drugs can present negative points for the treatment of TL, such as, their invasive route of administration, high cost, undesirable side effects ranging from fevers, nausea, vomiting, acute renal failure, alterations in cardiac functions and even abortion^{6,13,14}.

As a result, it is necessary to seek new therapeutic alternatives, and one of them involves natural products derived from plants of medical interest, which are of great importance in the search for new drugs with antileishmanial activity. This has been confirmed by several studies carried out in order to evaluate the antileishmanial activity found in extracts of medicinal plants over the years¹⁵⁻¹⁹. Numerous chemical compounds that have been isolated from plant extracts have demonstrated antileishmanial activity against the promastigote and amastigote forms of *Leishmania* sp. in *in vitro* biological assays. Antileishmanial activity of chemical groups has already been described, as in the case of terpenoids²⁰⁻²². This class of metabolites is present in large quantities in *C. odorata*, as indicated in phytochemical studies of this species, which presented limonoids as being predominant²³. In the present study, it was possible to isolate and characterize two limonoids: gedunin and mexicanolid; however, only mexicanolid had sufficient mass to be submitted to biological assays.

Experimental Section

Acquisition of wood residues

The woody residues of *C. odorata* were obtained from a window frame after the demolition of a forty-five-year-old building at the National Institute for Amazonian Research (INPA). After identification by the xylotheque, the residues were chipped, ground and subjected to a cold extraction in hexane and then with methanol, both for seven days each.

Isolation and characterization of compounds

The methanolic extract was subjected to successive chromatographic fractionations using open columns with different stationary phases (cellulose, sephadex-LH20 and 70-230 and 230-400 silica gel mesh). The compounds had their proposed structures based on NMR analyses ^1H , ^{13}C , DEPT 135, HSQC, HMBC, COSY and EMAR. The NMR spectra were obtained using a spectrometer (Bruker Fourier-300 model) (300 MHz - ^1H and 75 MHz - ^{13}C) at the Analytical Center of the Natural Products Chemistry Laboratory (CA-LTQPN) at INPA. The mass spectra were acquired through high resolution mass spectrometry (Bruker, microTOF-Q II model), time of flight analyzer, ESI source in positive mode; resolution of 17500 (FWHM); using the Compass program, version 4.1, for data control, acquisition and processing.

Promastigote activities of *Leishmania* spp.

The promastigote forms of *Leishmania* (V.) *guyanensis* (MHOM/BR/1995/IM4147) and *Leishmania* (L.) *amazonensis* (MHO/BR/2009/IM5584), cryopreserved in the Leishmaniasis and Chagas Disease Laboratory at INPA, were utilized. For the cultivation of the promastigote forms, RPMI 1640 medium was used, which was supplemented with inactivated bovine fetal serum (iFBS) and then 50 $\mu\text{g mL}^{-1}$ of antibiotic (gentamicin) was added, the solution was subsequently incubated at 25 °C for later use in bioassays²⁴.

Determination of antileishmanial activity and mean inhibitory concentration of the compounds

The evaluation of antileishmanial activity was made according to the inhibition of growth and mortality of promastigote forms. The methanolic extract and the limonoid were filtered and diluted in the culture medium at concentrations of 7.8 to 125 $\mu\text{g mL}^{-1}$. The positive control used was Pentacarinat® (pentamidine isethionate) at concentrations of 0.375 to 6 $\mu\text{g mL}^{-1}$ and the negative control was 1% dimethylsulfoxide (DMSO, Merck). The bioassay plate was incubated in an oven at 25 °C, for 24 to 72 hours. The assays were performed in triplicate and the mean number of living cells was used to calculate the values of the minimum inhibitory concentration (IC_{50}).

Statistical Analysis

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

Results and Discussion

Yields from the compounds

From 680 g of woody residues of *C. odorata*, a yield of 8.67 g of methanolic extract (COM) equivalent to 1.27% was obtained. From this extract, a predominant compound, namely mexicanolid (MEX), was isolated and characterized, which had a yield of greater than 1 g and, for this reason, it was used to perform the tests. In addition to this, there was another isolated compound, namely, the limonoid gedudin (GED); however, due to low mass, it could not be tested.

Structural determination of limonoids

Characterization of Gedunin (2 mg)

A white solid presented a pseudo molecular ion $[\text{M}+\text{H}]^+$ m/z 483.2396, which suggests the molecular formula $\text{C}_{28}\text{H}_{34}\text{O}_7$. In the HSQC, the doublet at δ 7.41 d (J = 1.4 Hz) correlated with the carbons at δ 141.09 (C-21) and δ 143.13 (C-23), while the triplet at δ 6.34 t (J = 1.4 Hz) correlated with

δ 109.81 (C-22), which indicates the presence of a furan ring. The singlet at δ 3.70 s correlated with δ 54.08 (C-15), while the singlet at δ 5.56 s correlated with δ 78.10 (C-17), which is characteristic of a δ -lactonic D ring. In the HMBC, the correlation of δ 5.56 (H-17) with δ 69.8 was noted, which was assigned to C-14, the only position at 3J to which it was possible to be assigned. The presence of the δ -lactonic D ring with an epoxy between C-14 and C-15 were confirmed by correlations observed in the HMBC. The 1H and ^{13}C NMR data were similar to those reported for gedunin (Figure 1) in *C. odorata* grafted with *Toona ciliata* var. *Australis*²³.

Characterization of Mexicanolid (1,009 mg)

This solid white compound presented a peak of the pseudo molecular ion $[M+H]^+$ m/z 469.2230, which is consistent with the molecular formula $C_{27}H_{32}O_7$. In HSQC, the broad singlet at δ 5.23 correlated with δ 80.76 (C-17), while in HMBC, it correlated with the signals at δ 141.60 (C-21), 133.82 (C-14), 120.32 (C-20), 109.96 (C-22), 37.98 (C-13) and 17.36 (Me-18). There were also other important correlations for the bicyclic system of the mexicanolid skeleton, i.e., the two diastereotopic hydrogens at δ 3.25 (H-30a) with the displacements at δ 133.82 (C-14); 125.33 (C-8) and 50.49 (C-9); and in δ 2.35 (H-30b) with the carbon signals δ 211.06 (C-3); 133.82 (C-14); 125.33 (C-8) and 58.00 (C-2). Signal correlations at δ 3.22 (H 2) with carbons at δ 213.04 (C-1); 211.06 (C-3); 133.82 (C-14); 125.33 (C-8) and 50.49 (C-9), thus confirming bicyclic closure. The spectral data from 1D and 2D NMR provided the identification of the mexicanolid (Figure 1) in the residues of *Cedrela odorata*²⁵.

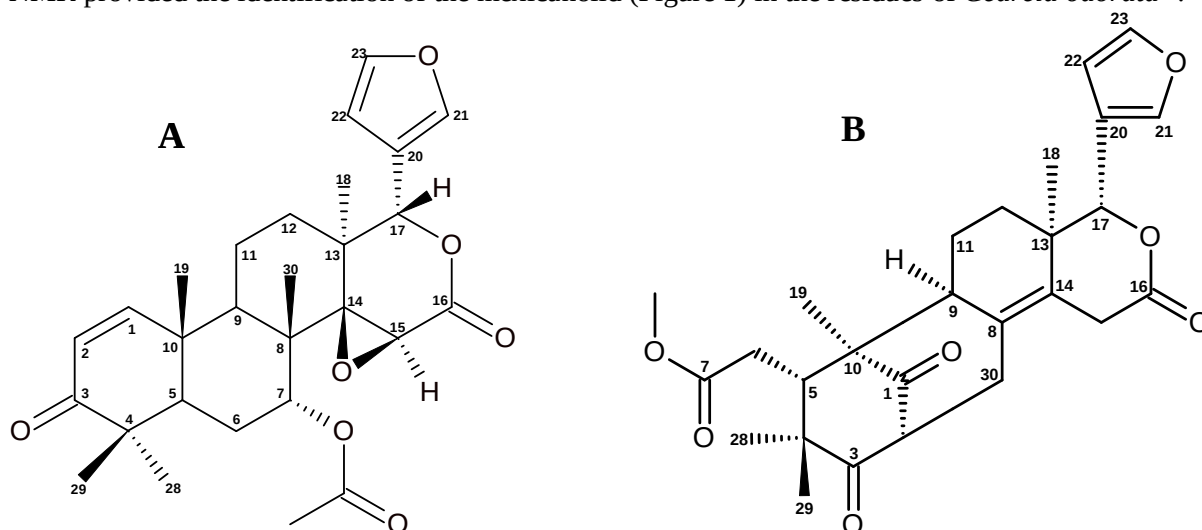
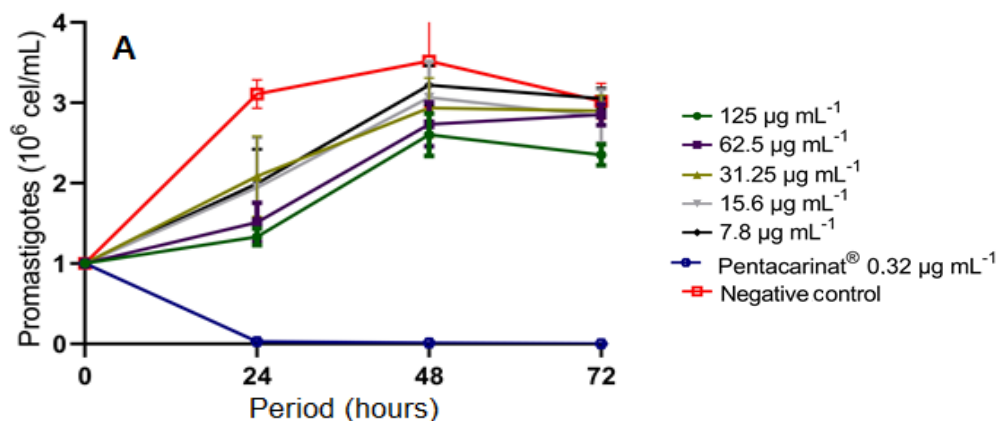


Figure 1 – Chemical structure of the limonoids gedunin (A) and mexicanolid (B) isolated from *C. odorata*.

At the two highest concentrations, the samples COM and MEX showed moderate results against *L. amazonensis* in the 24 h period, with a respective IC_{50} of 83.6 and $71 \mu g mL^{-1}$ (Figure 2). However, none of the samples demonstrated inhibitory activity against the species cited in the 72 h period, with an IC_{50} greater than $100 \mu g mL^{-1}$ (Table 1).



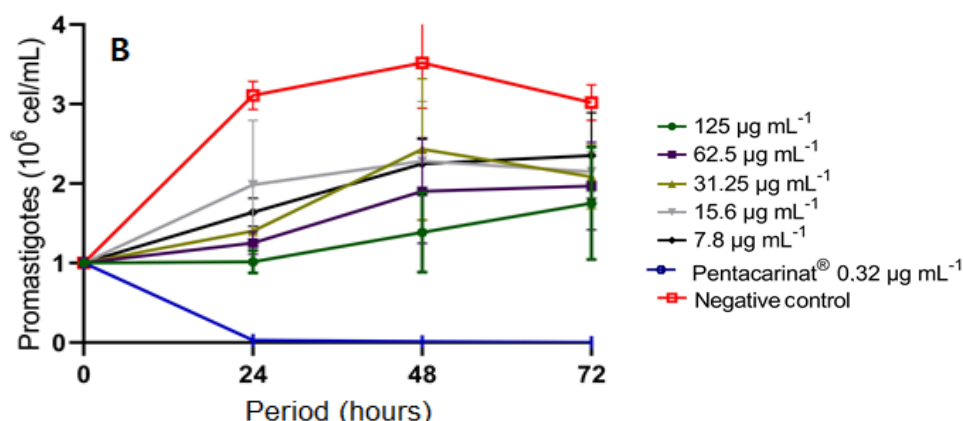


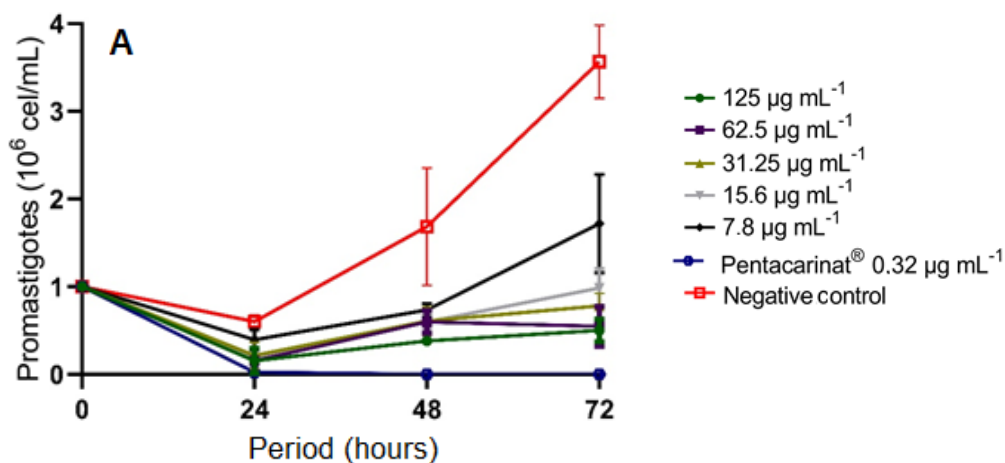
Figure 2 – Antileishmanial activity of the methanolic extract (A) and of the mexicanolid (B) against *L. amazonensis*.

The activities of the samples were classified according to the IC_{50} value, being: highly active ($IC_{50} < 10 \mu g mL^{-1}$), active (IC_{50} between $10-50 \mu g mL^{-1}$), moderately active (IC_{50} between $50-100 \mu g mL^{-1}$) and non-active ($IC_{50} > 100 \mu g mL^{-1}$)²⁶. Using these same criteria, we can measure the antileishmanial activity of the samples.

Table 1 – IC_{50} values of the methanolic extract (COM) and of the mexicanolid (MEX) from *C. odorata*.

Samples	$IC_{50} (\mu g \cdot mL^{-1})$	
	<i>L. amazonensis</i>	<i>L. guyanensis</i>
	72 h	72 h
COM	>100	12.9
MEX	>100	39.4
Pentacarinat®	< 0.63	< 0.63

In the biological assay of COM and MEX against the species *L. guyanensis*, the samples were active in the period of 24 to 72 h, mainly at concentrations of 15.6 to $125 \mu g mL^{-1}$ (Figure 3). The IC_{50} of the methanolic extract of *C. odorata* was equal to $12.9 \mu g mL^{-1}$ and the IC_{50} of the mexicanolid was equal to $39.4 \mu g mL^{-1}$ in the 72 h period (Table 1). Thus, methanolic extract showed greater activity than mexicanolid in the aforementioned period.



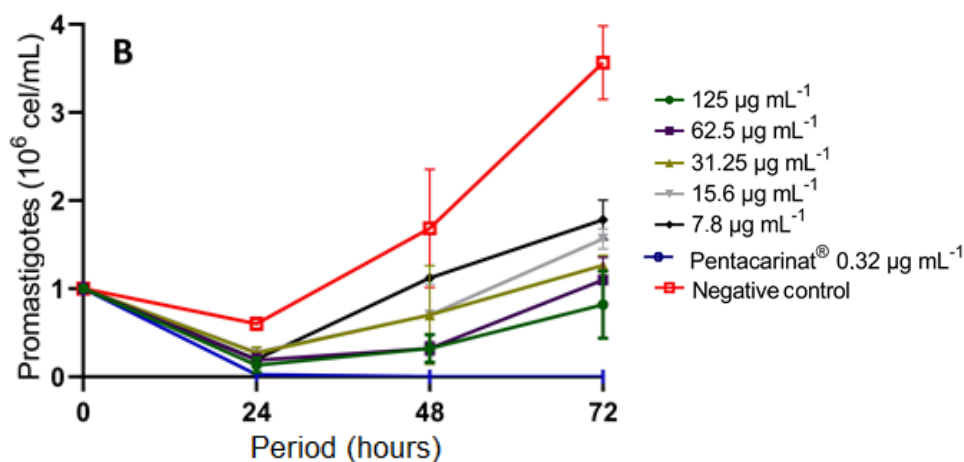


Figure 3 – Antileishmanial activity of the methanolic extract (A) and of the mexicanolid (B) against *L. guyanensis*.

This most significant result of the methanolic extract of *C. odorata* can be attributed to the fact that it is a complex of substances, mainly with the presence of different limonoids, which cause a combined action between the substances present in the sample and, consequently, the inhibition of parasitic growth²⁷. These results are consistent with previous studies in which it was possible to observe that the extracts of *C. odorata* showed significant inhibitory activity against *Leishmania infantum*, by inhibiting about 98.1% of the growth of the parasite at a concentration of 800 µg/ml). As such, this was the first study to report on the antileishmanial activity of the species *C. odorata*²⁸.

Final Considerations

The 1D and 2D NMR spectroscopic data, combined with high-resolution mass spectrometry, demonstrated similarity with the data reported in the literature, which permitted the characterization of the limonoids isolated in the methanolic extract. This shows that the chemical profile of wood from *Cedrela odorata* remain practically the same despite a long exposure to various natural factors, which possibly gives them resistance to xylophagous pests.

The COM and MEX samples tested against the promastigote forms of *L. guyanensis* showed the greatest inhibitory activity of parasitic growth and increased mortality, compared to the results of *L. amazonensis*. It is worth mentioning the importance of carrying out further studies to evaluate the cytotoxic activity in macrophages of the J774 line and against another evolutionary form of *Leishmania* sp. in order to determine a safe dose, as well as to confirm or not the efficacy of the samples for a possible alternative treatment of tegumentary leishmaniasis.

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