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Artigo

Antiparasitic Activity and Characterization of Lignans from the Ethanolic Extract of *Zanthoxylum monogynum* A. St-Hil. Leaves

Silva, F. B.; Trovó, M.; Gomes, A. L.; Mazotto, A. M.; Vermelho, A. B.; Martins, R. C. C.*

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Atividade Antiparasitária e Caracterização de Lignanas do Extrato Etanólico das Folhas de *Zanthoxylum monogynum* A. St- Hil

Resumo: O extrato etanólico das folhas da espécie vegetal *Zanthoxylum monogynum* A. St-Hil (Rutaceae) foi fracionado por partição líquido-líquido e por cromatografia de adsorção em coluna, resultando na caracterização das lignanas savinina, cubebina, 3,4-dimetoxil-3,4-metilenodioxicubebina e hinoquinina, que possuem diversas atividades biológicas já descritas. A identificação estrutural desses metabólitos foi feita por meio de análises de CG-EM e RMN 1D e 2D. O extrato também foi testado quanto a sua atividade contra o parasita *Trypanosoma cruzi*, causador da doença de Chagas, de grande incidência em regiões mais carentes do Brasil. A análise antiparasitária do extrato frente à forma epimastigota das cepas Y e Dm28c apresentou resultados de IC_{50} de 69,03 $\mu g.mL^{-1}$ e de 88,75 $\mu g.mL^{-1}$, respectivamente, considerados satisfatórios. Para propor um possível mecanismo de ação para a letalidade do extrato, conduziu-se um teste de zimografia com peptidases extraídas do parasita, tornando possível observar que o extrato inibiu a migração de metalopeptidases, essenciais para o seu ciclo de vida. Assim, *Z. monogynum* demonstra potencial para ser utilizada no tratamento da doença de Chagas.

Palavras-chave: *Zanthoxylum monogynum;* lignanas; *Trypanosoma cruzi;* doença de Chagas.

Abstract

Ethanolic extract from the leaves of *Zanthoxylum monogynum* A. St-Hil (Rutaceae) was fractionated by liquid-liquid partition and by column adsorption chromatography, resulting in the characterization of the lignans savinin, cubebin, 9*R*- and 9*S*-3,4-dimethoxyl-3,4-methylenedioxycubebin, and hinokinin, which have several biological activities already described in the literature. The structural identification of these metabolites was made by GC-MS and 1D and 2D NMR analysis. The extract was also tested for its activity against the parasite *Trypanosoma cruzi*, which causes Chagas disease, that has high incidence in individuals of the poorest regions of Brazil. The antiparasitic analysis of the extract against the epimastigote form of strains Y (domestic cycle) and Dm28c (wild and domestic cycle) presented IC_{50} results of 69.03 $\mu g.mL^{-1}$ and 88.75 $\mu g.mL^{-1}$, respectively, considered satisfactory. In order to propose a possible mechanism of action for the extract lethality, a gel zymography test was performed with peptidases extracted from the parasite. Results showed that the extract was able to inhibit migration of peptidases, which are essential for its life cycle. Thus, *Z. monogynum* leaves extract presents a promising potential for use in the treatment of Chagas disease.

Keywords: *Zanthoxylum monogynum*; lignans; *Trypanossoma cruzi;* Chagas disease.

* Universidade Federal do Rio de Janeiro, Instituto de Pesquisas de Produtos Naturais Walter Mors, CCS, Bloco H, Av. Carlos Chagas Filho 373, Ilha do Fundão, CEP 21941-902, Rio de Janeiro-RJ, Brasil.
roberto@nppn.ufrj.br
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Antiparasitic Activity and Characterization of Lignans from the Ethanolic Extract of *Zanthoxylum monogynum* A. St-Hil. Leaves

Fernanda B. da Silva,[a] Amanda L. Gomes,[b] Marcelo Trovó,[c] Ana M. Mazotto,[b] Alane B. Vermelho,[b] Roberto Carlos C. Martins,[a,*]

[a] Universidade Federal do Rio de Janeiro, Instituto de Pesquisas de Produtos Naturais Walter Mors, CCS, Bloco H, Av. Carlos Chagas Filho 373, Ilha do Fundão, CEP 21941-902, Rio de Janeiro-RJ, Brasil.

[b] Universidade Federal do Rio de Janeiro, Instituto de Microbiologia Paulo de Góes, CCS, Bloco I, Av. Carlos Chagas Filho 373, Ilha do Fundão, CEP 21941-902, Rio de Janeiro-RJ, Brasil.

[c] Universidade Federal do Rio de Janeiro, Instituto de Biologia, CCS, Bloco A, Av. Carlos Chagas Filho 373, Ilha do Fundão, CEP 21941-902, Rio de Janeiro-RJ, Brasil.

* roberto@nppn.ufrj.br

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1. Introduction

Zanthoxylum monogynum A. St-Hil (Rutaceae) is a plant popularly known as "laranjeira-do-mato", "tinguaciba", "limão-bravo" ou "limãozinho". This species is endemic in Brazil and occurs in the states of Alagoas, Bahia, Pará, Paraná, Espírito Santo, Goiás, Minas Gerais, Pernambuco, Rio de Janeiro, and São Paulo.[1] Until 2017, there was only one report in the literature describing the

composition and the biological activities of the essential oil from its leaves.[2] In 2017, Silva *et al.* described the cytotoxic activity of the essential oil showing that the best result of IC_{50} (11µg.mL^{-1}) was achieved when it was tested against HL-60 tumoral cell lines. Authors also reported a moderate antimicrobial activity of the essential oil against *Candida sp., Cryptococcus sp.*, and *Saccharomyces cerevisiae*[3]. These are, so far, the only reports in the literature for both chemical and biological studies with this plant.

Among the main targets of studies of plant biological activities are the Neglected Diseases (ND), which are diseases that cause great morbidity, but low mortality. According to WHO, there are 17 diseases considered as neglected, occurring mainly in countries that have a low-income population and, therefore, attracting no interest of the pharmaceutical industry.[4] These diseases reach about one billion people annually. Social inequality, lack of infrastructure, and health education are factors that favor their proliferation.[5]

In recent years there has been an increasing interest in searching for new drugs and ways of preventing NDs. Chagas disease is one of these ND's for which efforts for less expensive as well as less aggressive treatments have been currently highlighted. This disease is caused by the flagellate protozoan *Trypanosoma cruzi*, that infects humans, domestic and wild animals. The development of *T. cruzi* is divided into three stages. The first one is called epimastigote, and occurs in the digestive tract of the vector; the second, trypomastigote, is found in the vector, in the blood, and in the intercellular space of the host, and the third, the amastigote, is found in the proliferative form in host cells.[6]

The main route of transmission of Chagas disease is the bite of the insect known as "Barbeiro" (*Triatoma infestans,* order Hemiptera, family Reduviidae), which, during the bite, deposits feces contaminated with the trypomastigote form of *T. cruzi* in the host organism, leading to contagion.[7] The disease has two phases: acute and chronic. The initial phase is acute, it may either be asymptomatic or develop symptoms such as fever and edema at the site of inoculation. The chronic phase has an undetermined form, there are no symptoms, but the anti-*T.cruzi* antibody is present in the serum, and a determinate form, which occurs 10 to 30 years after the initial infection, being characterized by cardiac and digestive (megaoesophagus and mega colon) disorders.[8]

Several tests using natural sources substances have shown promising results in the fight against *T. cruzi*.[9] Lignans are a group of secondary metabolites that have presented great capacity of combating it, and some of them are reported as even more potent than benzanidazol, the medicine currently used in the treatment of Chagas disease.[10] Some extracts from *Zanthoxylum* species have already proven to have a potential trypanocidal activity. Methanolic extract of *Z. zanthoxyloides* stem barks showed trypanostatic effects *in vivo*, leading to the conclusion that the plant could completely eradicate the parasites. This resultreinforces that this medicinal plant can be a source of new bioactive substances in the fight against *Trypanosoma*.[11] In another study, carried out with ethanolic extract of the *Z. acuminatum* stem, lethality of the parasites with an IC_{50} of 17,0 µg.mL^{-1} was observed.[12] There is also a report of *in vitro* trypanocydal activity of the lignan methylpluviatolide, isolated from leaves of *Z. naranjillo*.[13]

Researches involving the understanding of the action mechanism are important to clarify the targets of the new drugs and their lethality on parasites. Taking into account the biochemical role of peptidases, enzymes capable of cleaving peptide bonds and regulating several biological processes, including host-parasite relationship, they are a potencial letal target.[14] There are several types of peptidases, of which cysteines peptidases and metallopeptidases may be important in the virulence factors of trypanosomatides. Several studies *in vivo* using animal models showed that the inhibition of these enzymes might result in the loss of virulence and in the reduction of the survival rate of these parasites.[15] Thus, the

aim of this study was to investigate the activity of an ethanolic extract and the organic fractions of *Zanthoxylum monogynum* A. St-Hil (Rutaceae) leaves against *T. cruzi* epimastigote forms – emphasizing the inhibition of its peptidases– and also provide a partial chemical profile of this plant extract, bringing out new data upon its chemical constitution and potential biological activity.

2. Materials and Methods

2.1. General procedures

The ^{1}H and ^{13}C NMR, including experiments in 2D ($_{2}JCH$ - HSQC, $_{3}JCH$ – HMBC) were recorded in a Varian MR-400 (400 MHz for ^{1}H e 100 MHz for ^{13}C) and a Varian VNMRSYS-500 (500 MHz for ^{1}H e 125 MHz for ^{13}C) spectrometers with $CDCl_3$ as solvent and TMS as standard. Chemical shifts (δ) are given in ppm and coupling constants (J) are given in Hertz (Hz) column. GC-MS analysis were performed on a GCMS-QP2010 Plus mass spectrometer equipped with a DB-5 column (30m x 250 mm x 0.25mm), with ionization energy of 70 eV. Injector temperature was set at 290°C. Column temperature programming used was from 60 °C to 300 °C with a 10 °C.min^{-1} heating rate. Helium was used as the carrier gas at a constant flow rate of 1mL.min^{-1} in the split mode (10:1); 2 mL of a 2mg.mL^{-1} dichloromethane solution of the compounds was injected for each analysis.

2.2. Collection of plant material

Fresh and undamaged leaves of *Zanthoxylum monogynum* were collected in May, 2013 at Itatiaia National Park, Rio de Janeiro State, Brazil. The species was identified by Dr. Marcelo Trovó (IB-UFRJ), and a voucher was deposited under the number MLO 601 (RB) in the herbarium (RBA) of the Universidade Federal do Rio de Janeiro (UFRJ).

2.3. Preparation of crude extract

Leaves were oven dried at 40 °C for 48 hours and grounded in a knives mill to a fine powder. Part of this powder (94.60 g) was extracted in a Soxhlet apparatus at 60 °C with 400mL of ethanol for a period of 2 hours, after which, solvent was evaporated under reduced pressure and the ethanolic extract (13.92 g) was obtained. An aliquot of it (500.0 mg) was separated for use in the anti *T-cruzi* assays.

2.4. Fractionation of the ethanolic extract

Aiming a selective fractionation, 7g of the ethanolic extract of *Z. monogynum* leaves was solubilized in 100mL of hydroalcoholic solution (30 % methanol) and submitted to a liquid-liquid partition with *n*-hexane, dichloromethane, and ethyl acetate, yielding three portions: ZMH (2.5 g), ZMD (1.4 g), and ZMA (715.4 mg), respectively.

ZMH portion was fractionated through a *flash* silica-gel (240-400 mesh) column chromatography under low pressure using as eluent systems *n*-hexane:ethyl acetate and ethyl acetate:methanol on an increasing polarity scale. It produced 155 portions, reunited by similarity according to the results observed in TLC plates revealed under UV light (258 and 365 nm) and sprayed with a 2 % H_2SO_4 solution followed by heating at 100°C. ZMH 77-110 (417.2 mg) submitted to a new *flash* silica-gel column chromatography under low pressure, using the same eluent system of the previous fractionation. As a result, 60 portions were collected from this experiment and compound **1** was isolated in the ZMH 77/25-28 (10.0 mg) portion.

ZMD portion was submitted to a silica-gel column chromatography and *n*-hexane:ethyl acetate and ethyl acetate:methanol on an increasing polarity scale were used as eluents. It produced 82 portions, grouped by similarity according to TLC analysis. From this fractioning, compound **2** was isolated in ZMD31-35 (197.0 mg), and compounds **3** and

4, in a mixture, in the ZMD39-43 (234.1 mg) portion.

2.5. Obtaining and cultivating *Trypanosoma cruzi*

Y (domestic cycle) and DM28c (wild and domestic cycle) strains of *T. cruzi* were used in the anti *T. cruzi* assays, material provided by the Ultrasctructure Celular Laboratory, Instituto Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil. Epimastigote forms of *T. cruzi* were maintained in PBHIL culture, medium enriched with 10 % fetal bovine serum (SFB) at 28 °C, as described in the literature.[16]

2.6. Evaluation of trypanocidal activity and determination of IC_{50}

Quantitative evaluation of the antiparasitic activity was made by successive microdilution assay in a 96-well plate for the determination of both Minimum Inhibitory Concentration (MIC) and IC_{50}. For this assay, the extract (ZMFEE) and portions ZMH, ZMD and ZMA were serially diluted from a stock solution of 100 $mg.ml^{-1}$ in DMSO to provide the following concentrations: 500, 250, 125, 62.5, 31.5, 15.6, 7.8 and 3.9 $\mu g.mL^{-1}$. Epimastigote forms of *T. cruzi*, 10^6 parasites per mL, were incubated in the absence and presence of different concentrations of the extract and bioactive substances. After the 5^{th} day of incubation at 28 °C, 50µL of resazurin (5 $mg.mL^{-1}$) was added to the wells, and the plate was incubated at 37 °C for 3 hours. After this time, it was read in a spectrophotometer at 490 and 595 nm.[16] Benzonidazol, as the positive control, was incubated in the same conditions of the samples. All assays were performed in triplicate, and standard deviation was calculated and used for comparison.

2.7. Evaluation of cytotoxic activity

Determination of viability in RAW264.7 macrophages was evaluated by incubation of cells with different extracts concentrations, the same used in trypanocidal assay. Controls were: negative control (culture medium without cells), positive control (culture medium with cells), control of DMSO (culture medium containing the highest DMSO concentration of dilution 0.2 %) with cells, turbidity control (culture medium + the compound evaluated at the same concentrations used without cells). After 48 hours of incubation at 37 °C and 5 % CO_2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added for spectrophotometric reading at 490 and 595 nm aiming the determination of cell viability (CC50). The assays were performed in triplicate. The selectivity index (SI) was calculated through the ratio between the cytotoxic concentration at 50 % (CC50) for cells (RAW 264.7) and IC_{50} for the *T. cruzi* cells.

2.8. Intracellular peptidase zymography gel

Intracellular peptidases are obtained by centrifugation of the parasites at 1,500g for 15 min at 4 °C. Cells are washed three times with sterile saline solution (NaCl 0,85) and lysed with 4x sample buffer with [0.32M Tris-HCl buffer, pH 6.8; glycerol 48 %, sodium dodecyl sulfate 8 %, bromophenol blue 0.002 %] in the proportion of 10^7 cells per 100µl of sample buffer, of which 10µL were applied per slot. Zymographic analysis of the cellular peptidases of the cultures was made on a 10 % polyacrylamide gel containing 0.2 % (w/v) copolymerized gelatin. The run was performed at 150V for 40 min in an ice bath. After this time, the gels were washed twice with 2.5 % (v/v) Triton X-100 for 15 min at 25 °C with shaking to remove SDS (sodium dodecyl sulfate). The gels were then incubated for 48 hours at 37 °C under the following conditions: 50mM phosphate buffer, pH 5.5

without inhibitors (positive control), phosphate buffer with 2 mM DTT (dithiothreitol) or 10 µM E-64 (for cysteine peptidases); 50 mM glycine-NaOH, pH 10.0 in the absence (positive control) or presence of 10 mM 1,10-phenanthroline (for metalopeptidases), or with 250 $\mu g.mL^{-1}$ of the test extract. The gels were stained with Coomassie blue R-250 0.2 % in methanol-acetic acid-water (50:10:40, v/v/v) and decolourised therein.

3. Results and Discussion

3.1. Identification of lignans

Four compounds were identified in the ethanolic extract of the *Z. monogynum* leaves, namely: savinin (**1**), cubebin (**2**), 3,4-dimethoxyl-3,4-methylenedioxycubebin (**3**), and hinokinin (**4**) (**Fig. 1**). The structural identifications were made by the analysis of the ^{1}H, ^{13}C, HSQC, COSY and HMBC NMR spectra and GC-MS data.

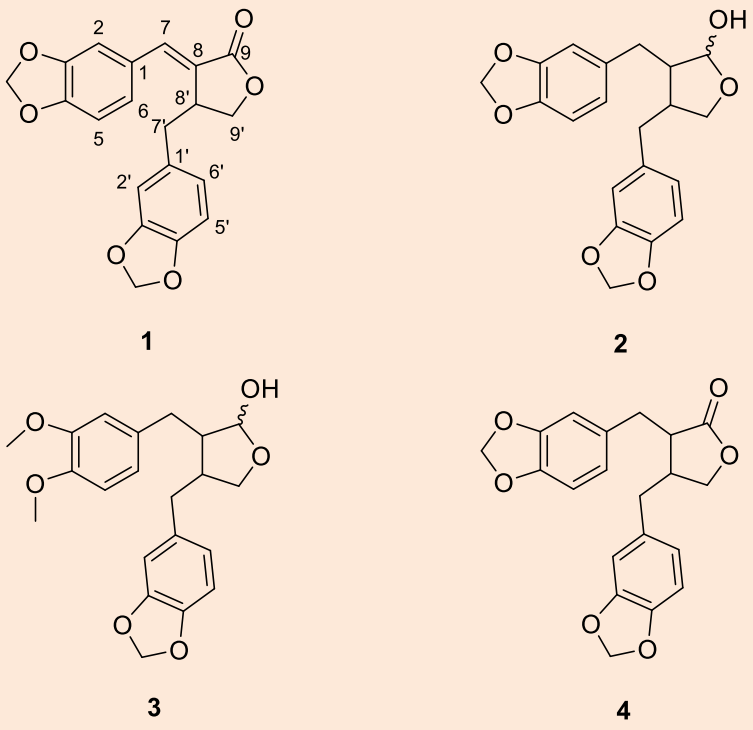

Figure 1. Lignans isolated from the ethanolic extract of *Z. monogynum* leaves

Compound **1** was isolated as a white crystal, soluble in dichloromethane. MS spectrum showed a molecular ion peak at *m/z* 352 [M+H], and additional analysis of the NMR data led to the proposition of the molecular formula $C_{20}H_{16}O_6$. In the ^{1}H NMR spectrum, a signal was observed at *δ* 2.59 (*dd*, *J* =14.2, 10.1 Hz, 1H) and δ 2.99 (*dd*, *J* = 14.3, 4.5 Hz, 1H) attributed to hydrogens attached to C-7'. A signal at δ 3.75 is attributed to H-8'. Two signals for two hydrogens at δ 4.12 and δ 4.23 were attributed to the hydrogens H-9a/b'. In δ 7.50 there is a singlet which corresponds to a hydrogen attached to a sp^2 carbon.

Aromatic hydrogens region of the NMR spectrum presents signals at *δ* 6.64 (*d, J*=8.0 Hz, 1H), δ 7.08 (*d, J* = 8.1 Hz, 1H), concerning to H-6 and H-6', at *δ* 6.74 (*d, J* = 7.8 Hz, 1H) and δ 6.88 (*d*, *J* = 8.1 Hz, 1H), concerning to H-5' and H-5, and at *δ* 6.67 (s, 1H) and *δ* 7.04 (*s,* 1H)

concerning to H-2' and H-2. In addition to them, two signals at δ 5.93 and δ 6.04 were also assigned to methylenedioxyl groups attached to the aromatic rings in C-3 and C-4 and C-3'and C-4'. The ^{13}C NMR spectrum showed a signal at δ_C 172.73 (C-9), characteristic of a carbonyl from a lactone. Signals at δ_C 126.25 (C-8) and 137,48 (C-7) correspond to *sp*^{2} carbons in a C=C bond. Signals were also observed at δ_C 69.97 (C-9', CH_2), δ_C 40.07 (C-8', CH) and δ_C 37.70 (C-7', CH_2). Signals at δ_C 101.20 and δ_C 101.88 are related to the carbons of the methylenedioxy group, and at δ_C 108.65 and δ_C 149.34, signals of the aromatic ring carbons.

Based on this data set, **1** was identified as savinin, a dibenzylbutyrolactone-type lignan, previously found in other species of *Zanthoxylum*, such as *Z. naranjillo, Z. pluviatile*, *Z. lemairie*, *Z. caudatum*, *Z. nitidum* and *Zanthoxylum capense*.[13,17-21] In addition, savinin exhibited strong insecticidal activity and cytotoxicity for HCT116 human colon carcinoma cells.[22]

Compound **2** was isolated as a white crystal, soluble in chloroform. Mass spectrum showed a molecular ion at *m/z* 356 [M+H] and analysis of the NMR data, made it possible to propose its molecular formula as $C_{20}H_{20}O_6$.

NMR data defined the sample as a mixture of isomers. ^{1}H NMR spectrum presented a singlet in δ1.65, attributed to a hydroxyl hydrogen. A wide singlet at *δ* 5.22 was attributed to a carbinolic hydrogen. Four double doublets at δ 3.79-4.00 e 3.57-4.09 were attributed to hydrogens H-9a/b', each pair of double doublets being attributed to an isomer. Signals between *δ* 2.00-2.43 are attributed to methyl hydrogens and between *δ* 2.43-2.76 hydrogens signals attributed H-7 and H-7'. In addition to them, a doublet in δ 5.92 assigned to the methylenedioxyl group and signals between δ 6.50 and δ 6.73 of aromatic hydrogens were also observed.

HSQC data analysis confirmed that the signal at δ 5.22 corresponded to two hydrogens, each one bounding to a single carbon, one signal at δ 103.26 and the other at δ 98.72. HSQC spectrum was also very important to assign the substitution patterns of the aromatic rings.

Based on this data set, compound **2** was identified as a mixture of cubebine isomers, a dibenzylbutyrolactone-type lignan.[23] In the presence of chloroform, the epimerization of cubebine occurs, which may explain the identity of the compounds in the mixture.[24] Cubebine is widely found in the plant kingdom, mainly in Piperaceae family.[25] Cubebine has already been found in *Zanthoxylum naranjillo*, and in this very study its trypanocidal and anti-inflammatory activity was described.[13] Its leishmanicidal, antimicrobial, antioxidant, vasodilator action, sexual stimulant, antimutagenic, and analgesic properties have also been reported.[23,26-31]

Compounds **3** e **4** were identified in a mixture soluble in chloroform. GC-MS data showed two peaks for this sample. One of them showed a molecular ion peak at *m/z* 372 [M+H], corrresponding to the molecular formula $C_{21}H_{24}O_6$, and the other presented a molecular ion peak at *m/z* 354 [M+H], corresponding to the molecular formula $C_{20}H_{18}O_6$.

Compound **3** is a 3,4-methoxylated isomer of cubebin (compound **2**). This was demonstrated by the similarity between the signals for both lignans, and the main difference observed in their ^{1}H NMR spectra was the presence of two intense singlets at δ 3.78 and δ 3.81. Besides, ^{13}C NMR spectrum presented four signals between δ_C 55.72 and 55.92 assigned to methoxyl groups, which were absent in the cubebin spectrum. Compound **3** was then identified as 3,4-dimethoxy-3,4-methylenedioxycubebin and had also been isolated in *Z. naranjillo*, together with cubebin and hinokinin.[13] It is also found in *Virola michelli* (Myristicaceae), *Aristolochia triangularis* (Aristolochiaceae), and *Piper chaba*.[32-34] So far, there are no reports describing biological activities for this compound in the literature.

Compound **4** also showed similarity of signals with cubebin and savinin in the NMR spectra. The structural difference between

cubebin and compound **4** is in the hemicetallic carbon (C-9), which is a carbonylic carbon in **4** instead of a carbinolic one, as seen in savinin. This was corroborated by the presence of a signal at δ_C 178.88, in the ^{13}C NMR spectrum, a value close to that presented by the savinin. Savinin differs from the compound **4** only by the type of bonding between C-7 and C-8. Signals related to methylenedioxyl groups at δ_C 100.83 and 100.94, such as those of cubebin and savinin, were also observed. Based on this data set, compound **4** was identified as hinokinin, a dibenzylbutyrolactone-type lignan, such as savinin.

Just like cubebin, hinokinin was isolated from *Piper cubeba* (Piperaceae), but it can also be obtained by oxidation of cubebin.[27] Hinokinin was previously identified in *Z. naranjillo* and *Z. monophyllum* extracts.[13,18] Literature reports antitumor, analgesic, anti-inflammatory, antimutagenic, antichagasic, and antibacterial activities for this lignan.[37]

All NMR data were compared to the literature to validate the assignment of the signals for both ^1H and ^{13}C. GC-MS data analysis was also very useful in providing mass spectra whose peaks were related to unique fragmentation patterns of the compounds, unequivocally confirming their structure.

3.2. Evaluation of anti *T. cruzi* activity of ethanolic extract ZMFEE and portions ZMH, ZMD and ZMA

According to growth inhibition results presented on table **1**, ethanolic extract of *Z. monogynum* leaves showed inhibitory effect in the highest concentration tested (500 $\mu g.mL^{-1}$). Calculated IC_{50} were 69.03 $\mu g.mL^{-1}$ for Y strain and 88.75 $\mu g.mL^{-1}$ for Dm28c strain (Tab. **4)**. Considering that a crude extract, which is a complex mixture of many substances, was tested, these results were considered satisfactory.

Liquid partitioning of ZMFEE in *n*-hexane, dichloromethane, and ethyl acetate yielded 3 portions named ZMH, ZMD, and ZMA, respectively. ZMA was not able to inhibit neither the growth of epimastigote forms of *T. cruzi* Y nor that of Dm28c. ZMD and ZMH portions presented inhibition bigger than 90 % and IC_{50} values of 78.31 $\mu g.mL^{-1}$ and 90.72 $\mu g.mL^{-1}$ for the Dm28c strain, and for strain Y the inhibition reached 100 % and IC_{50} values of 73.95 $\mu g.mL^{-1}$ and 35.53$\mu g.mL^{-1}$, respectively (Tabs. **2, 3,** and **4**).

Results also show a decrease in the activity of the portions (ZMH and ZMD) from the extract for the Y strain, although the values are not that far from the one calculated for ZMFEE. It might indicate that trypanocidal activity for this strain is not strongly affected by the fractionation.

Otherwise, parasitic activity of the extracts and portions against Dm28c strain showed that there was a significant decrease in IC_{50} for ZMH while ZMD showed a value very close to that calculated for ZMFEE. Differently from what was observed for the Y strain, fractionation showed ability to increase the activity, specially on the non-polar portion, leading to the hypothesis that the bioactive substances may be concentrated on it.

These values still show a high value for IC_{50} when compared to benzonidazol, but samples tested are a mixture of substances when compared to this control (3.98 $\mu g.mL^{-1}$), which is an isolated compound. Benzonidazole, a nitroimidazole used as a positive control in the tests, is the medicine used in Brazil for the treatment of Chagas desease, but it is not considered the most adequate drug for such treatment, as it is not active in the chronic phase and causes strong side effects to the patients, since it needsto be administered for long periods of time. These factors cause many patients to discontinue the treatment.[36]

Table 1. Inhibition (%) of the epimastigote forms of *Trypanosoma cruzi* (strains Y and Dm28c) by the ethanolic extract of *Z. monogynum* leaves

ZMFEE	**Y strain**	**Dm28c strain**
Concentration ($\mu g.mL^{-1}$)	% inhibition	% inhibition
500	100.00	92.12
250	100.00	94.77
125	87.01	90.78
62.5	42.97	31.25
31.25	22.53	14.62
15.63	11.40	0
7,81	0	0
3,90	0	0

Table 2. Inhibition (%) of the epimastigote forms of *Trypanosoma cruzi* (strains Y and Dm28c) by the dichloromethane portion (ZMD) of *Z. monogynum* leaves

ZMD	**Y strain**	**Dm28c strain**
Concentration ($\mu g.mL^{-1}$)	% inhibition	% inhibition
500	100.0	-
250	81.7	94.8
125	65.8	86.6
62.5	39.8	32.6
31.2	14.8	0
15.6	12.4	0
7.8	0	0
3.9	0	0

- undetermined

Table 3. Inhibition (%) of the epimastigote forms of *Trypanosoma cruzi* (strains Y and Dm28c) by the *n*-hexane portion (ZMH) of *Z. monogynum* leaves

ZMH	Y strain	Dm28c strain
Concentration (μg.mL^{-1})	% inhibition	% inhibition
500	100	-
250	100	96.8
125	79.0	87.5
62.5	68.4	33.4
31.25	56.5	0
15.63	26.4	0
7,81	6,7	0
3,90	0	0

- undetermined

Table 4. Trypanocidal activity of *Zanthoxylum monogynum* ethanolic extract and portions against *Trypanosoma cruzi*. Values are expressed as mean IC_{50} (μg.mL^{-1}) ± SD

Sample tested	IC_{50Y} (μg.mL^{-1}) ± SD	$IC_{50Dm28c}$ (μg.mL^{-1}) ± SD
ZMFEE	69.03 ± 0.34	88.75 ± 0.47
ZMH	73.95 ± 0.06	35.53 ± 0.21
ZMD	78.31 ± 0.14	90.52 ± 1.18
Benzonidazole (Positive control)	3.98 ± 0.06	3.98 ± 0.09

In order to suggest possible mechanisms of action for the activity of the extract, its effect on the intracellular peptidase activity of the parasite was also evaluated through zymography with gelatin substrate. The participation of cysteine-, serine-, metallo-peptidase in the host-cell interaction, morphological differentiation, and in the nutrition of the parasites has been reported[14]. Cruzipain was pointed as the major cysteine-peptidase from *T. cruzi*, being identified as a therapeutic target for treatment of Chagas disease. The acidic pH is the ideal condition for their activity, so pH 5.5 was used in the test. DTT was also used as the enzyme activator, and E-64 as its inhibitor. For metallopeptidase activity, pH 10 is an ideal value and, therefore, it was used in the test, together with its inhibitor, phenanthroline. The expression of matrix metallopeptidase (MMP9) has been described in *T. cruzi*[14].

In this analysis it was possible to detect two intracellular metallopeptidases of approximately 60 kDa and 45 kDa, and three cysteine peptidases of approximately 74 kDa, 69 kDa, and 47 kDa. It is possible to observe that *Z. monogynum* extract was able to inhibit these two metallopeptidases from both Y and Dm28c strains, nevertheless cysteine peptidase was not inhibited (**Fig. 2**). Considering these results, a possible mechanism of action of the extract of *Z. monogynum* may be suggested.

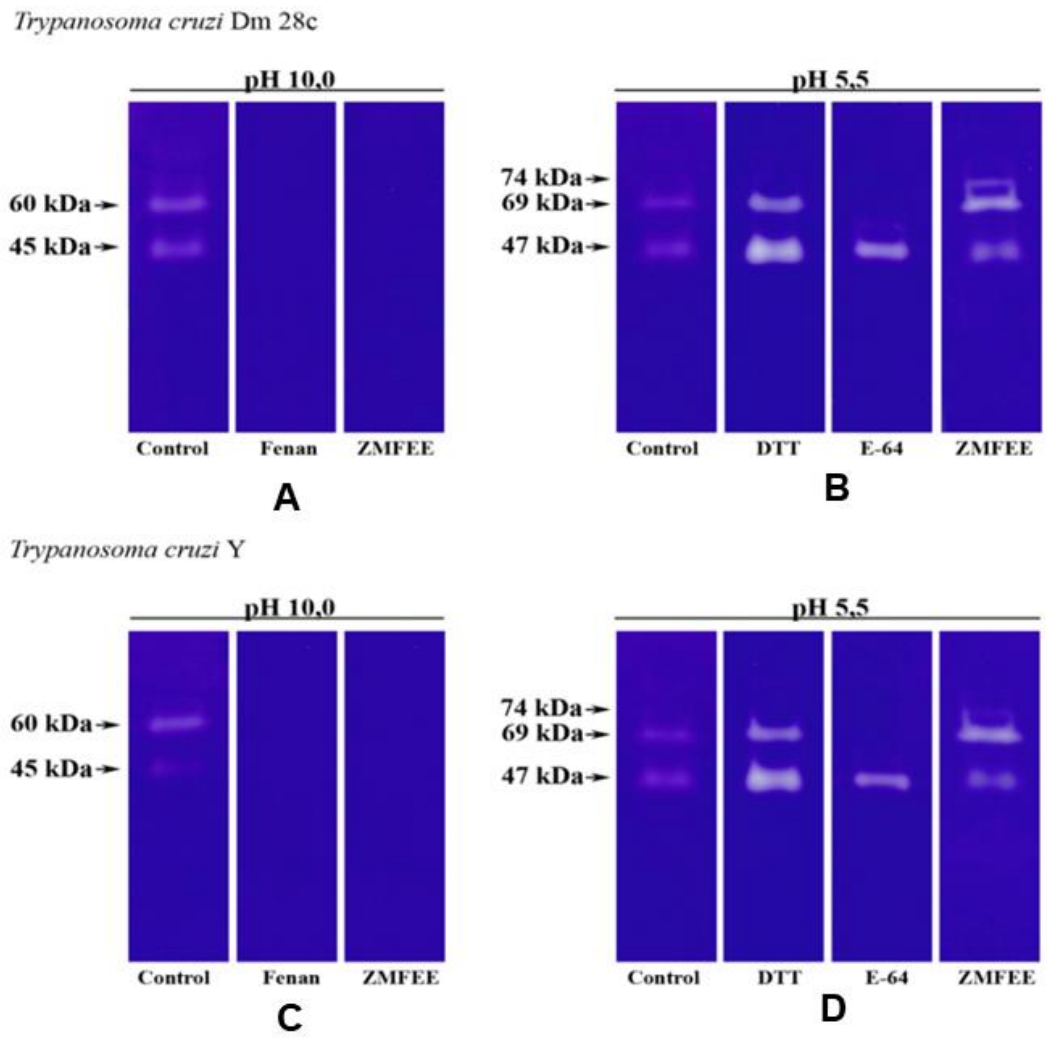

Figure 2. A) Zymography of metallopeptidase of *Tripanosoma cruzi* Dm28c in the presence and absence of the extract of *Z. monogynum* in pH10.0; B) Zymography of cysteine peptidase of *T.cruzi* Dm28c in the presence and absence of the extract of *Z. monogynum* in pH 5.5; C) Zymography of metallopeptidase of *T.cruzi* Y in the presence and absence of the extract of *Z. monogynum* in pH10.0; D) Zymography of cysteine peptidase of *T.cruzi* Y in the presence and absence of the extract of *Z. monogynum* in pH 5.5

E-64: cysteine peptidase inhibitor; DTT: cysteine peptidase activator; Fenan (1,10-orthophenanthroline), metallopeptidase inhibitor, Controle: positive control, ZMFEE: Z. monogynum* extract

Peptidases are crucial enzymes for the physiology and biochemistry of disease-causing etiological agents, performing an important role in the parasite-host interaction process in members of the Trypanosomatidae family. Inhibitors of these peptidases have been the subject of several studies aiming the development of chemotherapeutics of either natural or synthetic origin.[37] Among the six major classes of peptidases, the most commonly detected in trypanosomatides are metallopeptidases and cysteine peptidases.[38]

Metallopeptidases are widely found in trypanosomatids, including *T. cruzi*, which has a metallopeptidase homologous to gp63 of *Leishmania* (Tcgp63) of 60-65 kDa.[39] *T. cruzi* has 10 or more gp63 genes or molecules homologous to gp63 and their expression is increased in its amastigote phase, which indicates that this enzyme may play a role in

the survival and development of *T. cruzi* in the host. The cysteine peptidases, which are active, preferably under reducing and moderately acidic pH (5.0 to 6.5) conditions, catalyze the hydrolysis of polypeptide substrates.[40]

Anti *T. cruzi* studies with other species of *Zanthoxylum* have already been performed. *In vitro* tests have shown that the methanolic extract of *Z. zanthooloides* peels, popularly used in Guinea, against malaria presented IC_{50} 22.2 µg.mL^{-1}, against *T. cruzi*, and has no cytotoxic effect.[41] In Paraguay, the stem barks of *Z. chiloperone* is traditionally used in the treatment of Chagas disease. One study tested the effectiveness of leaves and stem extracts of this plant against the amastigote and trypomastigote forms of *T. cruzi. In vivo* tests have shown that both extracts have results similar to those of benzonidazole. The extracts tested also showed no cytotoxic effect against healthy human cells.[42]

Cubebin is an example of lignan whose trypanocidal activity has already been validated. Other lignans also have trypanosomicidal activity already recognised by previous studies.[25] This may indicate that lignans found in *Z. monogynum* may be responsible for the anti *T. cruzi* activity observed.

3.4. Evaluation of *in vitro* cytotoxic activity in mammalian cells

Cytotoxic concentration (CC_{50}) of the ZMFEE extract was evaluated *in vitro* in murine macrophages (RAW 264.7) and the selectivity index (SI) was calculated. The results showed that the CC_{50} of the extract was 444 µg/mL and the SI of 6.4 and 5.1 for *T. cruzi* Y and *T. cruzi* Dm 28c, respectively (Tab. 4).

Table 5. Cytotoxicity of ZMFEE extract and selectivity index (SI)

	ZMFEE	SI
CC_{50}	444 µg.mL^{-1}	-
IC_{50y}	69.03 µg.mL^{-1}	6.4
$IC_{50dm28c}$	5.75 µg.mL^{-1}	5.1

- Undetermined

Preliminary results showed that the extract is cytotoxic to murine cells (Tab. 4), but it is significatively more toxic to the parasites. Further tests are required to validate these results and compare them with benzonidazole, the drug currently used in the treatment of Chagas disease.

4. Conclusion

Genus *Zanthoxylum* (Rutaceae) is widely distributed throughout Brazil, but Brazilian native species are still poorly studied. Species of this genus accumulate biologically active substances that encourage studies. In this research, the extraction of *Zanthoxylum monogynum* leaves showed the presence of four dibenzylbutirolactone lignans and derivatives: savinin, cubebin, 3,4-dimethoxy-3,4-methylenedioxycubebin and hinokinin, suggesting a peculiar secondary metabolism acting in the species. Ethanolic extract of the leaves presented promising antiparasitic activity against the epimastigote form of *T. cruzi* strain Y and Dm28c. The best IC_{50} values were found in dichloromethanic and hexanic

portions, where these lignans were identified, and that might indicate that their correlation to the antiparasitic effect, although complementary bioguided studies must be held to support this hypothesis. These lignans also seem to be related to the activity of peptidases that may interfere in the life cycle of the parasite.

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