Chemical Constituents from Wood of *Zanthoxylum rigidum* Hum. & Bonpl. Ex Willd (Rutaceae) and Antileishmanial Activity

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Authors' contributions

This work was carried out in collaboration among all authors. Authors CHCS and MCS participated in phytochemical analysis. Author TANR participated in the evaluation of antileishmanial activity, acquisition and interpretation of data. Authors VCPS and MJJ participated in interpretation of NMR spectral and MS data. Author VCPS authored the project and supervised its execution and the drafting of this paper. Author PTSJ participated in the discussion of the data presented herein as well as the drafting of the manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

In this study was reported the structural elucidation by means of nuclear magnetic resonance (1D and 2D), FT-IR and mass spectrometry of lupeol (1), a mixture of the steroids campesterol (2), β-sitosterol (3) and stigmasterol (4), sesamin (5), *trans*-dimethylmatairesinol (6), *trans*-methylpluviatolide (7), quercetin (8), and D-mannitol (9) isolated from wood of *Zanthoxylum rigidum*. The lignans 5, 6 and 7 were tested for their ability to inhibit the proliferation of the promastigote forms of *Leishmania braziliensis* and *Leishmania chagasi*. Only sesamin (5) and *trans*-methylpluviatolide (7) showed moderate inhibitory activity with IC_{50} = 22.4 and 33.6, SI = 3.87 and 2.41 and CC_{50} = 86.9 and 81.2, for *L. braziliensis* and *L. chagasi*, respectively, compared to the positive control (amphotericin B).

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1. INTRODUCTION

The *Zanthoxylum* genus belongs to the Rutaceae family and comprises about 549 species distributed worldwide, although it is mainly found in tropical and temperate regions [1,2]. In addition, this genus has been used worldwide to treat different conditions such as snakebites, stomach problems, skin lesions, inflammation, and parasitic diseases [3-7]. Studies have shown that *Zanthoxylum* genus exhibit a range of biological activities such as antichagasic, trypanocidal, antiplasmodial, anti-HIV, anti-inflammatory, anti-helminthic, gastroprotective as well as cytotoxic [8-10]. In the Pantanal, species of *Zanthoxylum* in general, are known popularly as "mamica-de-porca" (*pig's nipple*) or "mamica de cadela" (*sow's nipple*) due to the particular shape of its thorn and are used in folk medicine, in the form of decoction, against different types of inflammation, rheumatism and skin stains [8]. The species *Zanthoxylum rigidum* Humb. & Bonpl. ex Willd., is used as forage, apiculture and civil construction [11]. Previous studies have described isolation of

terpenes, steroids, flavonoids, alkaloids and sugars from the stem bark and cyclopeptides from the leaves of the *Z. rigidum* Humb. & Bonpl. ex Willd (Rutaceae) [7,12]. Leishmaniasis is a parasitic disease caused by a species of protozoan of the genus *Leishmania*, of which more than 20 species are known to be pathogenic to man. The various variations of the disease are transmitted by female mosquitoes of the genus *Phlebotomus* that act as disease-transmitting agent. According to WHO, 90% of the cases of visceral leishmaniasis are registered in Bangladesh, Brazil, Nepal, India and Sudan; 90% of the cases of mucocutaneous leishmaniasis occur in Brazil, Bolivia and Peru and 90% of the cases of cutaneous leishmaniasis occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria [13]. The aim of the present study is to report the compounds (Fig. 1) isolated from wood of *Z. rigidum*, as well as to investigate the inhibition of the proliferation of different forms of *Leishmania* promastigotes by lignans sesamin (**5**), *trans*-dimethylmatairesinol (**6**) and *trans*-methylpluviatolide (**7**).
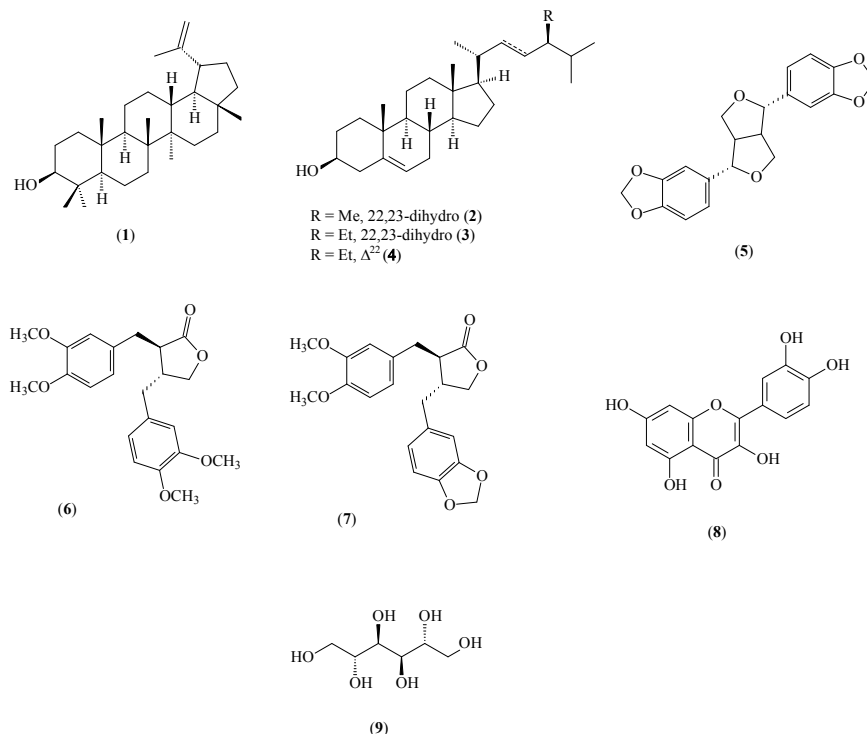
Fig. 1. Structures of the compounds isolated from wood of *Z. rigidum*

2. MATERIALS AND METHODS

2.1 General Experimental Procedures

Low-resolution mass spectra were performed using a gas chromatograph coupled to mass spectrometry (GC-MS) model GCMS - QP2010 Plus Shimadzu, using an ion trap and ionization by electron impact at 70 eV. IR spectrum was determined on an IRAffinity-1S Fourier Transform Infrared spectrophotometer from Shimadzu Corporation. The Nuclear Magnetic Resonance spectra, ^{1}H and ^{13}C (including 2D experiments) were recorded on Bruker Magnet System AscendTM 500 (^{1}H: 500 MHz and ^{13}C: 125 MHz) spectrometers using $CDCl_3$ and DMSO-d_6 as solvents and TMS as an internal reference. In column chromatographic separations, silica gel 60 (70-230 *mesh*, Merck) and Sephadex LH-20 (GE Healthcare) were used; in the analytical and preparative thin layer analyzes, silica gel 60 PF$_{254}$ (Merck) with adequate granulation and development under UV light (254 and 365 nm), acid vanillin or exposure to iodine vapors was used.

2.2 Plant Material

The wood of *Z. rigidum* was collected in Cuiabá-Mato Grosso, Brazil, on the road to the Manso Lake, km 20. A voucher specimen is deposited at the Central Herbarium of the Federal University of Mato Grosso under number 24985.

2.3 Extraction and Isolation

The plant material (1.3 kg) was powdered and successively extracted with methanol at room temperature, providing 172 g of methanolic crude extract. This extract was subjected to solid-liquid partition on silica gel 60 with hexane, ethyl acetate and methanol, resulting the residues hexane (10.5 g), ethyl acetate (23.4 g) and methanol (91.3 g). The hexane residue (10.5 g) was chromatographed on silica gel 60 column (70-230 *mesh*) eluted with hexane and dichloromethane, gradually increasing the polarity to obtaining of 67 fractions (150 mL). These fractions were analyzed by TLC and reunited in 19 fractions (15 mL). In the fraction 8 (45.4 mg) a white amorphous solid precipitated and after filtration in acetone, provided the triterpene lupeol (**1**, 451.8 mg). The fraction 6 (67.7 mg) provided another white solid corresponding to the mixture of the steroids campesterol (**2**), β-sitosterol (**3**) and stigmasterol (**4**). The ethyl acetate residue (23.4 g) was subjected a new fractionation on a silica gel 60

(70-230 *mesh*) and microcrystalline cellulose (1:1) column, using hexane, ethyl acetate and methanol with increasing of polarity affording 77 fractions (15 mL). These fractions were analyzed by TLC and grouped according to similarity of retention factor (R$_f$) in 13 fractions (15 mL), and after this process, there was the precipitation of a crystalline solid in fraction 5, which provided sesamin (**5**, 102 mg). Fractions 7-9 (4.2 g) were combined by similarity and subjected to filtration on Sephadex LH-20 in methanol and 53 fractions were obtained. After analysis by TLC, in the fractions 27-31 (650 mg) was observed the presence of two major compounds that corresponded to *trans*-methylpluviatolide (**6**, 22.7 mg) and *trans*-methylpluviatolide (**7**, 97.8 mg), separated through a preparative TLC eluted in the $CHCl_3$: MeOH system (9:1). The methanol residue (91.3 g) was subjected to the classic column on silica gel 60 (70-230 *mesh*) eluted with hexane, chloroform, ethyl acetate and methanol afforded 105 fractions (10 mL), combined according to their similarity in 15 fractions (15 mL). Fraction 3 yielded a yellowish amorphous precipitate which was recrystallized in acetone and provided again the mixture of steroids (**2**-**4**, 420 mg). In fractions 5-7, precipitate a crystalline solid in the form of needles occurred, again providing lupeol (410.3 mg). In fraction 13, a yellow precipitate formed that was filtered through $CHCl_3$, providing quercetin (**8**, 273 mg). From fraction 15 there was a spontaneous precipitation of a white crystalline solid, D-mannitol (**9**, 610.4 g).

2.4 Evaluation of Antileishmanial Activity

Murine macrophages of the J774 A.1 strain (ATCC CR-107), macrophage/monocyte cell type, derived from an adult female BALB/c sarcoma were used and cultured in RPMI-1640 cell culture medium, supplemented with streptomycin antibiotics (10 mg/mL), penicillin (6 mg/mL) and kanamycin (2 mg/mL), and 10% fetal bovine serum (FBS), kept at 37ºC and 5% CO_2. Stationary phase promastigote forms of *L. (V.) braziliensis* and *L. (L.) chagasi* were used and maintained in Schneider culture media supplemented with streptomycin antibiotics (10 mg/mL) and penicillin (6 mg/mL), and 20% fetal bovine serum (FBS), kept at 26ºC in a BOD incubator. The parasites used were obtained from cell culture from 6 to 7 days of growth when the promastigote forms are in the stationary phase. The *in vitro* cytotoxicity assay was performed to determine the inhibitory concentration of 50% (IC$_{50}$) using 1×10^5 of

promastigote forms of *L. braziliensis* and *L. chagasi*, maintained in Schneider medium, supplemented with fetal bovine serum (FBS) at 26ºC incubated in 96-well plates with the standard drug amphotericin B (10 µg/mL) and the compounds **5**, **6** and **7**. These compounds were tested in concentrations between 0.5 to 250 µg/mL dissolved in Schneider culture medium and 0.1% DMSO. As a negative control, the parasites were grown only in Schneider and Schneider medium containing the diluent of the compounds (DMSO 0.1%). The viability of the promastigote forms was evaluated based on the MTT metabolism, being the same proportional to the absorbance value generated in a spectrophotometer. After 72 hours, incubation at 26ºC, 50 µL of MTT solution (2 mg/mL) were added to each well and then incubated for 4 hours at 24ºC. After this period, the plate was centrifuged at 5,000 rpm for 7 minutes and the supernatants from each well were removed and 100 µL DMSO was added. The formazan crystals were dissolved by shaking and the absorbance was determined by an ELISA reader at 540 nm. The data were plotted on a linear regression curve and the results expressed in IC_{50} [14]. This experiment was carried out in duplicate. Macrophages J774 A.1, were grown in RPMI-1640 medium, supplemented with streptomycin antibiotics (10 mg/mL), penicillin (6 mg/mL) and kanamycin (2 mg/mL), and 10% fetal bovine serum (FBS), maintained at 37ºC and 5% CO_2, this culture after acquiring semi-confluence, was washed once with the Hanks buffer solution, trypsinized and the cells counted in a Neubauer chamber, adjusting the number cells to $2x10^5$ cells/mL in complete RPMI medium. From this suspension, the cells were seeded in 96-well plates and incubated at 37ºC and 5% CO_2 for 24 hours. Next, different concentrations of the compounds **5**, **6** and **7** were prepared in concentrations between 0.5 to 250 µg/mL, to which the cells were added, being incubated at 37ºC and 5% CO_2 for 24 hours. As a negative control, the cells were cultured containing the 0.1% DMSO diluent. Doxorubicin (10 mg/mL) was used as a positive control in this bioassay. The cytotoxic concentration 50% (CC_{50}) of each compound was evaluated using the alamar blue redox indicator [15]. After the incubation period, the culture medium was removed and then 20 µL of alamar blue and 180 µL of complete RPMI medium were added. After 6 hours of incubation, the absorbance was read at 570 nm (oxidized state) and 595 nm (reduced state) in the ELISA reader (BIORAD 640) and the visual reading of the plate was done by the redox

indicator, where the blue color oxidized represents cell death and the lilac color represents viable cells. The data were analyzed using a linear regression curve and the results were expressed as CC_{50}, being considered CC_{50} cytotoxic <50 µg/mL [16]. The experiments were carried out in triplicate. The selectivity index (SI) was calculated considering the ratio between CC_{50} and IC_{50} for each compound tested (CC_{50}/IC_{50}).

3. RESULTS AND DISCUSSION

The hexane residue from wood of *Z. rigidum* resulted in the isolation of lupeol (**1**), a mixture of the steroids campesterol (**2**), β-sitosterol (**3**) and stigmasterol (**4**). The ethyl acetate residue resulted in the identification of the lignans sesamin (**5**), *trans*-dimethylmatairesinol (**6**) and *trans*-methylpluviatolide (**7**). From methanol residue, lupeol (**1**), the steroids mixture (**2**-**4**), the flavonoid quercetin (**8**) and great abundance of the carbohydrate D-mannitol (**9**) were obtained. The structures of lupeol (**1**), quercetin (**8**) and D-mannitol (**9**) were established by analysis of ^{1}H and ^{13}C NMR spectra, comparisons using standards by TLC analysis and literature data [17-19]. For confirmation of the steroids mixture, campesterol (**2**), β-sitosterol (**3**) and stigmasterol (**4**), a GC-MS analysis was conducted. The analysis of the mass fragments from each component provided the peaks corresponding to the molecular ions at *m/z* 400 (campesterol), *m/z* 412 (stigmasterol) and *m/z* 414 (β-sitosterol) [20]. The structures of the lignans sesamin (**5**), *trans*-dimethylmatairesinol (**6**) and *trans*-methylpluviatolide (**7**), were defined based on the analysis of ^{1}H and ^{13}C (DEPT-Q) and HMQC and HMBC spectroscopic data, MS, IR and comparison with literature data [21,22].

Lupeol (1). White *amorphous* solid; $C_{30}H_{50}O$; EIMS *m/z* 426 [M][+.]; IR (KBr film) v_{max} (cm^{-1}) 2953, 2872, 2298, 1456, 1377, 1017; NMR [1]H [500 MHz, CDCl₃, δ (ppm), *J* (Hz)]: 0.67 (*m*, H-5), 0.76 (*m*, H-24), 0.79 (*s*, H-28), 0.83 (*s*, H-25), 0.90 (*m*, H-23), 0.94 (*s*, H-27), 1.03 (*s*, H-26), 1.18 (*m*, H-22), 1.25 (*m*, H-9), 1.36 (*m*, H-16), 1.39 (*m*, H-7), 1.40 (*m*, H-11), 1.45 (*m*, H-18), 1.52 (*m*, H-6), 1.60 (*m*, H-15), 1.62 (*m*, H-12), 1.65 (*m*, H-1), 1.66 (*m*, H-13), 1.67 (*m*, H-2), 1.71 (*s*, H-30), 1.92 (*m*, H-21), 2.40 (*m*, H-19), 3.20 (*dd*, H-3), 4.55 and 4.70 (*d*, 1.9, H-29); NMR [13]C [125 MHz, CDCl₃, δ (ppm)]: 14.6 (C-27), 15.5 (C-24), 16.0 (C-26), 16.1 (C-25), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 21.0 (C-11), 25.2 (C-12), 27.4 (C-2), 27.6 (C-15), 28.0 (C-23), 29.8 (C-21),

34.3 (C-7), 35.5 (C-16), 37.2 (C-10), 38.0 (C-13), 38.8 (C-4), 38.9 (C-1), 40.8 (C-8), 40.5 (C-22), 42.9 (C-17), 43.2 (C-14), 48.3 (C-18), 48.0 (C-19), 50.4 (C-9), 55.3 (C-5), 78.9 (C-3), 109.2 (C-29), 151.0 (C-20) [17].

Sesamin (5). Colorless crystal; $C_{20}H_{18}O_6$; EIMS *m/z* 354 [M]$^{+}$; IR (KBr film) ν_{max} (cm^{-1}) 2850, 1500, 1442, 1193; NMR ^{1}H [500 MHz, CDCl$_3$, δ (ppm), *J* (Hz)]: 3.07 (*m*, H-1,5), 3.90 (*dd*, *J* = 9.15, 3.70, H-4/8) and 4.25 (*dd*, *J* = 9.15, 6.70, H-4/8), 4.74 (*d*, *J* = 4.40, H-2/6), 5.97 (*s*, H-9/10), 6.83 (*dd*, *J* = 8.2, 1.5, H-6'/6"), 6.80 (*d*, *J* = 7.90, H-5'/5"), 6.87 (*d*, *J* = 1.50, H-2'/2"); NMR ^{13}C [125 MHz, CDCl$_3$, δ (ppm)]: 54.3 (C-1/5), 71.7 (C-4/8), 85.8 (C-2/6), 101.1 (C-9/10), 106.5 (C-2'/2"), 108.2 (C-5'/5"), 119.4 (C-6'/6"), 135.1 (C-1'/1"), 147.1 (C-4'/4"), 148.0 (C-3'/3") [21].

trans-Dimethylmatairesinol (6). White amorphous solid; $C_{22}H_{26}O_6$; EIMS *m/z* 386 [M]$^{+}$; IR (KBr film) ν_{max} (cm^{-1}) 2920, 1776, 1591, 1489, 1157; NMR ^{1}H [500 MHz, CDCl$_3$, δ (ppm), *J* (Hz)]: 4.14 (*dd*, *J* = 6.40, 4.10, H-9a) and 3.91 (*dd*, *J* = 7.20, 5.10, H-9b), 2.98 (*dd*, *J* = 14.10, 5.40, H-7'a) and 2.94 (*dd*, *J* = 14.10, 6.60, H-7'b), 2.55 (*m*, H-8), 2.65 (*m*, H-8'), 2.70 (*m*, H-7), 3.88 (*s*, H-10), 3.87 (*s*, H-13), 3.85 (*s*, H-11), 3.84 (*s*, H-12), 6.50 (*d*, *J* = 2.0, H-2), 6.57 (*dd*, *J* = 8.10, 1.90, H-6), 6.67 (*dd*, *J* = 8.10, 1.9, H-6'), 6.70 (*d*, *J* = 1.95, H-2'), 6.77 (*d*, *J* = 8.30, H-5'), 6.79 (*d*, *J* = 8.20, H-5); NMR ^{13}C [125 MHz, CDCl$_3$, δ (ppm)]: 34.5 (C-7'), 38.2 (C-7), 41.1 (C-8), 46.6 (C-8'), 55.8 (C-13), 55.9 (C-12), 56.0 (C-11), 60.0 (C-10), 71.3 (C-9), 111.0 (C-5), 111.3 (C-5'), 111.7 (C-2), 112.3 (C-2'), 121.4 (C-6'), 120.5 (C-6), 130.2 (C-1'), 130.4 (C-1), 147.9 (C-3/3'), 149.0 (C-4/4'), 178.7 (C-9') [22].

trans-Methylpluviatolide (7). White amorphous solid; $C_{20}H_{20}O_6$; EIMS *m/z* 370 [M]$^{+.}$; IR (KBr film) ν_{max} (cm^{-1}) 2920, 1774, 1598, 1510, 1160, [500 MHz, CDCl$_3$, δ (ppm), *J* (Hz)]: 2.53 (*m*, H-8), 2.61 (*m*, H-8'), 2.88 (*dd*, *J* = 14.05, 7.15, H-7), 2.98 (*dd*, *J* = 14.05, 5.2, H-7'), 3.86 (*s*, H-12), 3.88 (*s*, H-11), 3.92 (*m*, H-9), 5.96 (*d*, *J* = 1.45, H-10), 6.45 (*d*, *J* = 1.45, H-2), 6.62 (*dd*, *J* = 7.35, 1.40, H-6/6'), 6.69 (*d*, *J* = 1.80, H-2'), 6.74 (*d*, *J* = 7.65, H-5), 6.79 (*d*, *J* = 8.10, H-5'); NMR ^{13}C [125 MHz, CDCl$_3$, δ (ppm)]: 34.6 (C-7'), 38.3 (C-7), 41.1 (C-8), 46.5 (C-8'), 55.8 (C-12), 55.9 (C-11), 71.2 (C-9), 101.1 (C-10), 108.3 (C-5), 109.5 (C-2), 111.3 (C-5'), 111.6 (C-2'), 122.3 (C-6), 120.6 (C-6'), 130.4 (C-1'), 131.5 (C-1), 146.3 (C-4'), 146.5 (C-3), 147.9 (C-4), 149.0 (C-3'), 178.5 (C-9') [22].

Quercetin (8). Yellow solid; IR (KBr film) ν_{max} (cm^{-1}) 3385, 3282, 1667, 1615, 1429, 1210; NMR ^{1}H [500 MHz, DMSO-d_6, δ (ppm), *J* (Hz)]: 6.20 (*d*, 1.95, H-6), 6.40 (*d*, 1.95, H-8), 6.92 (*d*, 8.5, H-5'), 7.65 (*dd*, 2.05, 8.5, H-6'); 7.75 (*d*, 2.05, H-2'); NMR ^{13}C [125 MHz, CDCl$_3$, δ (ppm)]: 93.0 (C-8), 97.8 (C-6), 103.1 (C-10), 114.6 (C-2'), 114.8 (C-5'), 120.3 (C-6'), 122.7 (C-1'), 135.8 (C-3), 144.8 (C-3'), 146.6 (C-4'), 147.4 (C-2), 156.8 (C-9), 161.1 (C-5), 164.2 (C-7), 175.9 (C-4) [18].

D-Mannitol (9). White crystalline needles; IR (KBr film) ν_{max} (cm^{-1}) 3400, 3300, 2900, 1100, 1050; NMR ^{1}H [500 MHz, DMSO-d_6, δ (ppm), *J* (Hz)]: 3.54 (*dd*, 11.0, 5.8, H-1'/6'), 3.64 (*ddd*, 8.4, 5.8, 2.5, H-2/5), 3.68 (*m*, H-3/4), 3.75 (*dd*, 11.0, 2.5, H-1/6); NMR ^{13}C [125 MHz, DMSO-d_6, δ (ppm)]: 63.2 (C-1/6), 69.5 (C-3/4), 70.9 (C-2/5) [19].

The antileishmanial activity of the compounds **5**, **6** and **7** against *L. brasiliensis* and *L. chagasi* strains was evaluated. These compounds were used in concentrations ranging from 0.5 to 250 μg/mL and analyzed at different time periods (24, 48 and 72 hours) in order to determine the inhibitory concentration against the tested *Leishmania* strains. The results obtained showed that among the tested compounds, only **5** and **7** with IC$_{50}$ of 22.4 μg/mL and 33.6 μg/mL, showed the greatest efficacy against the promastigote forms of *L. braziliensis* and *L. chagasi*, respectively and are shown in Table 1. The standard drug amphotericin B, used as a positive control, had an inhibitory effect on the proliferation of promastigote forms, reducing this parameter to 0.38 μg/mL and the diluting agent DMSO (0.1%) did not change the viability and proliferation of promastigote forms. Alamar blue was used as an effective tool to assess the metabolic activity and proliferation of cell lines. In this bioassay, only the reduction of resazurin to resorufin is quantified, which revealed that all compounds showed low toxicity compared to the cell line J774. A1. The results provided the values of CC$_{50}$ and was verified that the compounds **5** and **7** could be tested in concentrations below 50 μg/mL. The selectivity index evaluated the selectivity of the compounds against the parasite, and the highest values were observed for **5** (3.87) and **7** (2.41), indicating a greater efficacy and safety of these compounds against *L. braziliensis* and *L chagasi* compared to compound **6**. These results showed that the increase in antiproliferative activity may be related to the presence of methylenedioxy groups in the structure, according to reports found in the literature [23,24].

Table 1. Antileishmanial effect of the lignans from wood of *Z. rigidum*

Compound	CC_{50} (µg/mL) J774 A.1	*L. chagasi*		*L. brasiliensis*	
		IC_{50} (µg/mL)	SI	IC_{50} (µg/mL)	SI
5	86.9 (78.01-91.05)	93.0 (82.3-107.3)	0.93	22.4 (16.81-32.4)	3.87
6	68.6 (53.25-79.14)	102.3 (97.2-112.5)	0.67	93.3 (84.1 – 98.6)	0.73
7	81.2 (75.58-89.22)	33.6 (24.27-42.78)	2.41	112.5 (96.3-124.1)	0.72

4. CONCLUSION

The isolated pure compounds were characterized as lupeol (**1**), a mixture of the steroids campesterol (**2**), β-sitosterol (**3**) and stigmasterol (**4**), sesamin (**5**), *trans*-dimethylmatairesinol (**6**), *trans*-methylpluviatolide (**7**), quercetin (**8**) and D-mannitol (**9**) by FT-IR, MS, ^{1}H and ^{13}C NMR (1D and 2D). Compounds **5**, **6**, **8** and **9** were identified for the first time in the wood of *Z. rigidum*. The isolated lignans **5**, **6** and **7** were tested for their antileishmanial activity against different species of *Leishmania*, and **5** and **7** showed better results having IC_{50} values of 22.4 and 33.6 µg/mL, and SI 3.87 and 2.41, against the promastigote forms of *L. braziliensis* and *L. chagasi*, respectively. The results for CC_{50} showed that the compounds (**5**) and (**7**) can be used in concentrations below 50 µg/mL. These results can be useful in the development of natural antileishmanial agents for the eradication of cutaneous and visceral leishmaniasis, a neglected disease that is still very common in tropical and subtropical countries, based on the chemistry of bioactive compounds from plants in the Brazilian Pantanal.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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