

Bioactive Neolignans from the Leaves of *Piper rivinoides* Kunth (Piperaceae)

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Abstract: *Piper rivinoides* is a Brazilian Piperaceae species of a little research from a chemical and biological point of view. The crude hydroalcoholic extract from leaves, the *n*-hexane fraction and pure isolated compounds from the leaves were evaluated against two strains of *Candida albicans* as well as *Leishmania amazonensis* and *L. chagasi*. Bioguided purification of the *n*-hexane fraction lead to isolation and chemical characterization of a mixture of two benzofuran neolignans, 2-(4'-methoxyphenyl)-3-methyl-5-(*E*)-propenylbenzofuran (**1**) and eupomatenoid-3 (**2**) as well as the pure compounds: eupomatenoid-5 (**3**), (2*R*,3*R*)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran (**4**), eupomatenoid-6 (**5**) and conocarpan (**6**). The first approach in quantification found with basis on quantitative nuclear magnetic resonance showed that the *n*-hexane fraction contains about 29.8% of eupomatenoid-5 (**3**), 7.6% of eupomatenoid-6 (**5**) and 5.5% of conocarpan (**6**). The biological results showed conocarpan as the most active compound with less cytotoxicity than the other tested compounds. Our results suggest conocarpan as owner of the best scaffold model of the four neolignans tested for new promising antimicrobial agents against the tested parasites. The high content of neolignans in the *n*-hexane fraction stands out *P. rivinoides* as an important source of bioactive neolignans.

Keywords: Piperaceae; *Piper*; benzofuran neolignans; *Leishmania*; *Candida*. © 2016 ACG Publications. All rights reserved.

1. Introduction

Species from Piperaceae family are generally shrubs and rarely trees, and are widely distributed in tropical and subtropical regions [1,2]. In Brazil they can be found from North to South, including in

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the Atlantic Forest and in the Amazonia region [2-5]. *Piper nigrum* L. is the most representative species of the family and is known worldwide due its use as a condiment and for its medicinal properties [6].

Ethnobotanical studies on the genus *Piper* have shown many species with medicinal applications, mainly to treat wounds, swellings, skin irritations [7], as expectorant for the treatment of cough, bronchitis and asthma [8], and to treat arthritic conditions [9].

Phytochemical investigations of Piperaceae species have shown different classes of secondary metabolites, including alkaloids, amides, lignoids, chromenes, lactones, terpenes and flavonoids [6, 10-15]. Neolignans are a class of secondary metabolites frequently isolated from the genus *Piper* such as those with benzofuran, dihydrobenzofuran and tetrahydrofuran structures [9, 10, 12, 16-20]. Studies accomplished with benzofuran neolignans showed a great variety of biological activities, including insecticidal [17], anti-trypanosome [20], antibacterial [21], anti-neuroinflammatory [22] and antitumoral [23].

Candidiasis represents the major opportunistic mycosis in humans, and it is often associated with the patient clinical condition, for example, it is very severe in immunocompromised patient. Fungal infections are normally difficult to treat and the available useful drugs have generally microbiostatic action that depends, in great part, on innate immune defense to solve the disease. *Candida albicans* is a member of normal flora that colonizes skin and mucosal surfaces of healthy people, occurring commensally in the gastrointestinal tract, oral and vaginal mucosal, often causing superficial infections but also able to disseminate and cause invasive candidiasis [24-29]. Fluconazole has been the main therapy for the treatment of superficial candidal infections, but its overuse, especially among immune compromised patients, has been implicated in the increase of candidal resistance and the shifting in the regular flora of candidiasis from *C. albicans* to less susceptible species. By this mean it is very important to investigate new anti-candidiasis agents [30].

Leishmaniasis is caused by more than twenty species of trypanosomatids of the genus *Leishmania* and can affect animals and humans in tropical and sub-tropical regions [31]. The cutaneous leishmaniasis is a disease characterized by a heteroxenic cycle evolution that affects mainly skin or mucous membranes and is distinguished by the presence of ulcerative lesions [32,33]. The parasites migrate to the lymphatic vessels, causing lesions throughout the skin to another hyperergic pole (cutaneous mucosa leishmaniasis) that causes the impairment and destruction of mucosal and cutaneous structures and frequently producing facial mutilation. Despite recent advances, the treatment of leishmaniasis continues to be unsatisfactory. Pentavalent antimonials remain the first-line treatment for this infection in most endemic areas, despite their limitations, such as high toxicity and increased drug resistance [33-36].

Thus, the development of new drugs that can improve the therapeutic strategies to treat fungal infections by *Candida* and *Leishmania* is urgent. In this context, plant extracts can offer a source of a great variety of new compounds and scaffolds for bioactive molecules [37] [38].

In this study we evaluated the antifungal (candidiasis) and the anti-parasitic (leishmaniasis) activities of the crude hydroalcoholic extract from *Piper rivinoides* leaves, its *n*-hexane fraction and four neolignans. We also isolated two more neolignans in mixture but in few amount (5.5mg). In addition to the biological assays we did a first approach (since the methodology was not validated) on quantification based on nuclear magnetic resonance (qNMR) of three neolignans in the bioactive *n*-hexane fraction.

2. Materials and Methods

2.1. Plant Material

Leaves of *Piper rivinoides* Kunth (Piperaceae) were collected in October 2010, in the city of Rio de Janeiro, Brazil, and was identified by Dr. Elsie Franklin Guimarães. A voucher specimen was deposited in the Rio de Janeiro Botanical Garden Herbarium under the number RB 554171.

2.2. Phytochemical procedures and analysis (General)

Silica gel (Merck, 60-200 mesh) and Sephadex LH-20 (Pharmacia) were used for column chromatographic separation. Silica gel plates 60 F₂₅₄ layers (Merck) were used for analytical TLC, visualized with UV 254nm or 365nm and after with cerium (IV) sulphate reagent. Absolute alcohol, methanol, *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol were purchased from Tedia (Brasil). Melting points were determined on a Koffler instrument. For internal standard issues dimethylbenzaldehyde (Sigma-Aldrich) resonance at 9.85 ppm (aldehyde proton, 1H, s) was used.

2.3. Extract preparation and isolation of pure compounds

Leaves of *P. rivinoides* were dried at room temperature and fragmented in a knife mill affording 230 g. The extract was prepared by static maceration with a mixture of ethanol: water (9:1, v/v) at room temperature, filtered, and concentrated under vacuum at 40°C. The obtained hydroalcoholic extract was then lyophilized yielding 20 g of an amorphous green solid residue. The extraction procedure was performed at Institute of Pharmaceutical Technology (Farmanguinhos), Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. The crude hydroalcoholic extract was tested against two *Candida* strains (one fluconazole-resistant), and against *Leishmania amazonensis* and *L. chagasi*. Due the positive activity against *Candida* strains and *L. chagasi* (Tables 1 and 2), 19 g of the hydroalcoholic extract was suspended in a mixture of water: methanol (7:3) and successively partitioned with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol.

2.4. Bioguided isolation of secondary metabolites

The *n*-hexane fraction obtained from the crude hydroalcoholic extract was assayed against *Candida* and *Leishmania* strains as a bioguided fractionation implies. The positive antimicrobial activity motivated us to do the phytochemical investigation of the *n*-hexane fraction. This fraction (2 g) was submitted to silica gel (60-200 mesh) column chromatography (CC) purification eluted with an increasing polarity gradient of *n*-hexane-ethyl acetate-methanol. Separation procedure afforded 5.5 mg of a mixture of two compounds identified as 2-(4'-methoxyphenyl)-3-methyl-5-(*E*)-propenylbenzofuran (**1**) and eupomatenoid-3 (**2**), eluted with pure *n*-hexane; 140.7 mg of eupomatenoid-5 (**3**) eluted in *n*-hexane/ ethyl acetate (99:1). The compound (2*R*,3*R*)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran (**4**, 10.1 mg) was obtained after separation on Sephadex LH-20 (eluted with pure methanol) from a mixture with eupomatenoid-5 (**3**). An amount of 72.0 mg of eupomatenoid-6 (**5**) was obtained from the CC eluted with *n*-hexane/ ethyl acetate (95:5), and finally, 20.7 mg of (+)-conocarpan (**6**) were obtained from the CC eluted with *n*-hexane/ ethyl acetate (90:10). The pure compounds **3**, **4**, **5**, **6** were submitted to the biological tests since we obtained pure and in suitable amounts. Isolation and purification as well as NMR experiments were conducted at Institute of Natural Product Research Walter Mors (IPPN), Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Compounds were identified by spectroscopic analysis (1D and 2D ¹H and ¹³C NMR) and by comparison with literature data [17, 39-41].

2.5. Nuclear Magnetic Resonance Spectroscopy (NMR)

Pure compounds obtained from leaves of *P. rivinoides* were analyzed by ^1H (400 MHz) and ^{13}C NMR (75 MHz) and recorded on a VNMRs 400 spectrometer (Varian Inc., Palo Alto, CA, USA). The chemical shifts were determined in CDCl_3 and DMSO-d_6 , using TMS as the internal standard. The signals of the NMR analyses were compared to the literature data [17, 39-41]. For a first quantitative purpose in order to estimate the amount of neolignans, pulse width, relaxation delay and the acquisition delay were optimized through a series of experiments. Data acquisition was performed through 512 scans, with 22.00 seconds for relaxation delay and 7.99 seconds were used for acquisition time. We set the relaxation delay as being five times the largest T1 found through Inversion Recovery pulse sequence. The equation applied was: $mS = (IS/IIS) \cdot (MMS/MMIS) \cdot (NS/NIS) \cdot (1/mIS)$ (eq. 1); where mS, IS, MMS and NS, stands for sample mass, intensity of a specific signal, molecular mass and number of spins a specific signal; and mIS, IIS, MMIS and NIS, stands for internal standard mass, intensity of a specific signal, molecular mass and number of spins a specific signal [42]. For internal standard issues dimethylbenzaldehyde resonance at 9.85 ppm (aldehyde proton, 1H, s) was used.

2.6. Fungus strains and culture conditions

Two *Candida albicans* strains were used in this study. One of them was obtained from the Institute of Microbiology Paulo de Góes at the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. This yeast was isolated from the oral mucosal of a patient with acquired immunosuppression (HIV+), and it is fluconazole resistant, namely PRI [43]. The other strain was obtained from a human bronchomycosis at the American Type Culture Collection under the designation ATCC10231.

2.7. Leishmania strains and culture conditions

Leishmania amazonensis Josefa strain (MHOM/BR/75/Josefa), originally isolated from a human cutaneous leishmaniasis and *L. chagasi* PP75 strain (MHOM/BR/PP75) promastigote forms were maintained by weekly transfers in 25 cm² culture flasks with Schneider's insect medium (SIM) (Sigma Aldrich® St. Louis/ USA), pH 7.2, supplemented with 10% fetal bovine serum (FBS) and gentamicine (80 µg/mL) at 26°C.

2.8.1. Minimal Inhibitory Concentration (MIC) - *Candida albicans*

The MIC was determined according to the Clinical and Laboratory Standards Institute (CLSI) third edition M27-A3 methodology (2008) for microdilution. The inoculum was prepared by suspending the cells in RPMI 1640 medium with *L*-glutamine without sodium bicarbonate (Sigma Chemical®) to correspond to a final inoculum's concentration of 5.0×10^3 cfu/mL. All compounds were considered water insoluble and therefore a stock solution were prepared using (DMSO). Nevertheless, all dilutions were set in accordance with CLSI methodology third edition M27-A3 (2008) and the final preparation in test did not exceed 0.5% of DMSO, which has been previously considered innocuous to the yeast [44,45]. Serial dilutions of all the compounds were prepared in a range of 100 to 0.09 µg/mL. Fluconazole was acquired commercially from Farmacopa Pharmaceuticals (Rio de Janeiro, Brazil), and used as antifungal control in concentrations ranging from 250 to 0.24 µg/mL. The microplates were incubated at 37°C for 48 h. The minimum concentration that inhibited completely (100%) and 50% of growing was defined as MIC and IC₅₀, respectively, and were calculated with the SigmaPlot (version 8) program. The results are expressed as the means ± standard deviation of three independent experiments.

2.8.2. Minimal Inhibitory Concentration (MIC) – *Leishmania*

Protozoa in exponential growth phase (10^6 parasites/mL) were plated in the presence of micro serial dilutions of compounds, starting from a concentration of 0.5 mg/mL to 0.97 μ g/mL. After incubation at 26°C for 72 h, cell viability was measured by the colorimetric method reduction of MTT salt [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] [46]. Minimum Inhibitory Concentration (MIC) and Inhibitory Concentration of 50% (IC₅₀) of extracts and pure neolignans on *L. amazonensis* and *L. chagasi* were calculated by Microsoft Excel[®] linear regression. The results are expressed as the means of three independent experiments and results were expressed in means \pm standard deviation.

2.9. Cytotoxicity to mammal cells

To evaluate the possible toxicity of the lignans to mammalian cells, a cytotoxicity assay was performed using L929 fibroblasts (mouse), RAW monocyte/macrophage (mouse) cells and VERO kidney epithelial cells (monkey). Cells were grown in Eagle's minimum essential medium (Eagle-MEM) or Dulbenco modified Eagle's minimum essential medium supplemented with 10% (v/v) of FBS, glutamine (0.03 mg/mL), garamycin (50 μ g/mL), fungizone (amphotericin B) (2.5 mg/mL), NaHCO₃ solution at 0.25% and HEPES (10 mM). Cell cultures were prepared in a 96 well microtiter plates and incubated at 37°C in a 5% CO₂ atmosphere. Several concentrations of compounds (range from 100 to 0.09 μ g/mL) were placed in contact with the confluent cells monolayers and incubated at 37°C in a 5% CO₂ atmosphere for 72 h. Cytotoxicity was evaluated by the neutral red dye-uptake method described elsewhere by Borefreund and Puerner [47]. Briefly, cells were incubated in the presence of 0.01% neutral red solution for 3 h at 37 °C in a 5% CO₂ atmosphere, and then fixed with 4% formalin in PBS, pH 7.2. Dye incorporated by the viable cells was eluted using methanol: acetic acid: water solution (50:1:49), and dye uptake was determined by measuring the optical density of the solution at 490 nm. The concentration that caused a 50% reduction in the number of viable cells was defined as the 50% cytotoxic concentration or CC₅₀. Selectivity index of the pure compounds was determined as the ratio of CC₅₀ versus IC₅₀ of each microorganism (*Candida* and *Leishmania* strains). All experiments were repeated at least 3 times, all systems were performed in triplicate sets and results were expressed in means \pm standard deviation.

2.10. Statistical Analysis

The data were analyzed statistically using Student's *t*-test. P values of 0.05 or less were considered to be statistically significant. Data were analyzed by SigmaPlot (version 8) program.

3. Results and Discussion

3.1. Compounds isolation and chemical characterization

The leaves crude hydroalcoholic extract and its *n*-hexane fraction showed activity against two strains of *Candida albicans* (ATCC 10231 and fluconazole resistant PRI) and *L. chagasi*, under the assayed conditions (Table 1). The dichloromethane fraction showed to be less effective against the parasites. The other fractions (ethyl acetate and *n*-butanol) demonstrated no activity. The high antimicrobial activity of the *n*-hexane fraction led us to investigate this sample in order to isolate the metabolites that are held responsible for the biological activity. The *n*-hexane fraction was submitted to sequential chromatographic separations that allowed the isolation of a mixture of two benzofuran neolignans, characterized as 2-(4'-methoxyphenyl)-3-methyl-5-(*E*)-propenylbenzofuran (**1**) and

eupomatenoid-3 (**2**). Eupomatenoid-5 (**3**), (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran (**4**), eupomatenoid-6 (**5**) and conocarpan (**6**) were also isolated (see experimental) (Figure 1). All compounds, including the mixture of 1 and 2, were identified by comparative analysis of their ^1H and ^{13}C NMR spectroscopic data [17, 39-41]. Compound 4 NMR data were compared to compound 6 allowing the chemical identification since they are different by the presence only of one methoxyl group. The activity of **3**, **4**, **5** and **6** on the microorganisms' viability is summarized on Table 1.

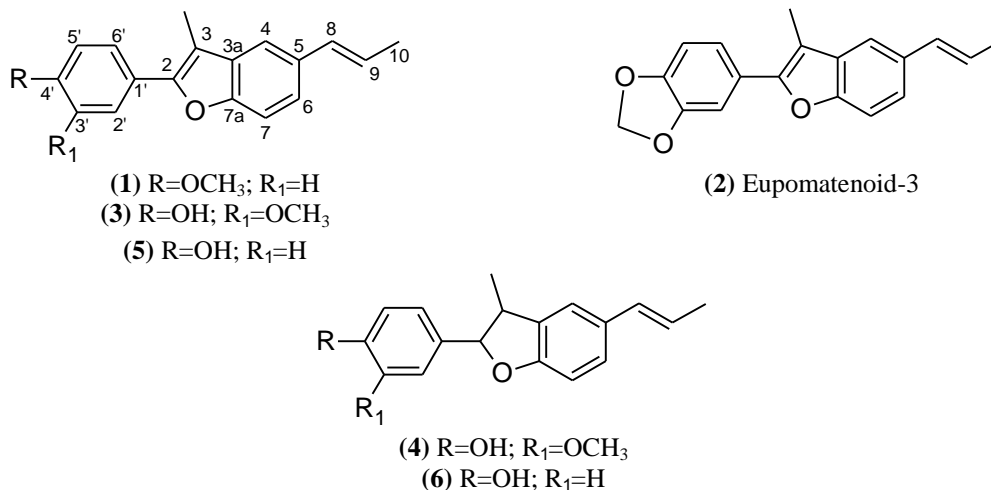


Figure 1. Neolignans isolated from the leaves of *Piper rivinoides*: (**1**): 2-(3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran; (**2**): eupomatenoid-3; (**3**): eupomatenoid-5; (**4**): (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran; (**5**): eupomatenoid-6; (**6**): conocarpan.

3.2. Antimicrobial activity and cytotoxicity

Among the purified neolignans, conocarpan (**6**) was the most effective compound ($p < 0.05$) against yeast grown reaching the lowest MIC and IC₅₀ levels against both *C. albicans* strains. Indeed, conocarpan (**6**) was more effective in inhibit the *C. albicans* PRI strain than fluconazole and this neolignan showed to be only about thirteen times less effective (IC₅₀ = 0.0736±0.0086 mM) in inhibit the *C. albicans* ATCC10231 strain than the standard drug (IC₅₀ = 0.0056±0.0003 mM) (Table 1). There was difference ($p < 0.05$) between the anticandidal activity of compounds **4** and **5** (Table 1). These results showed that a hydroxyl group at position 4' as well as a dihydrobenzofuran ring is very important to increase the activity. It can be seen that a methoxy group at 3' and the benzofuran group can decrease the activity ($p < 0.05$). By this mean, eupomatenoid-5 (**3**) that contains a methoxy group in position 3' and a benzofuran ring showed no activity in both *Candida* strains (Table 1).

In recent years, a number of plants of *Piper* genus have been found to have antifungal activity against several yeasts strains, which has allowed the isolation of the active principles followed by their characterization [48-51]. De Campos and co-workers (2005) [52] demonstrated the activity of conocarpan against *C. albicans* ATCC 10231 strains with MIC values around 30 µg/mL (0.11 mM) corroborating with our findings. Also, studies on the antimicrobial properties of conocarpan have shown that it is active against *C. albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae* as well as against the dermatophytes *Microsporum gypseum* and *Tricophyton mentagophytes* [48]. Pessini and co-workers (2005) [21] reported the antimicrobial activity of another *Piper* species, *P. regnelli*, and pure neolignans. The hexane fraction showed significant antibacterial (*Staphylococcus aureus* and *Bacillus subtilis*) activity while chloroform soluble fraction was less active than the hexane fraction. Besides, the other polar fractions showed no activity against the tested microorganisms.

Again, these findings are in agreement with ours. The pure compound conocarpan was active with MIC of 6.25 µg/mL (0.02 mM). Pessine and colleagues (2005) [21] also showed that methoxyl benzofuran neolignans were not active against the tested microorganisms. These data can reinforce that is very important the free hydroxyl group at position 4' and a dihydrobenzofuran group in the structure. However, Johann and colleagues (2009) [49] showed that eupomatenoid-6 and conocarpan, isolated from *Piper abutiloides*, strongly inhibited the *Candida glabrata*, *C. parapsilosis* and *C. krusei* growth, but were not effective in inhibiting the *C. albicans* ATCC 18804 growth. These data differ from ours since we demonstrated that conocarpan (**6**) was highly effective in inhibiting the growth of *C. albicans* ATCC10231 and a clinical strain resistant to fluconazole. In fact, it is known that different strains show different responses to drugs [53].

Regarding the protozoa, conocarpan (**6**) was the most successful compound to inhibit ($p < 0.05$) the growth of both species of *Leishmania* (CE_{50} *L. amazonensis* = 0.04 mM and *L. chagasi* = 0.09 mM). These results represent for the first time the efficient activity of conocarpan against *Leishmania* species viability and growth. Eupomatenoid-6 (**5**) and (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran (**4**) also showed activity on both parasite strains followed by eupomatenoid-5 (**3**) (Table 2). These results suggest that a free hydroxyl group at position 4' is very important to increase the antileishmanial activity. The benzofuran or dihydrobenzofuran group not to matter for the leishmanicidal activity as it is to the antifungal and antibacterial. Barata and co-workers (2000) [54] showed that neolignans isolated from *Virola* species (Myristicaceae) were able to inhibit the growth of *Leishmania donovani* promastigotes and axenic amastigotes. Garcia and colleagues (2003) [55] reported that the leishmanicidal property of *Piper regnellii* is related to the presence of the neolignan eupomatenoid-5 from the crude extracts and from the chloroform soluble fractions. In other study, eupomateino-5 showed to be more selective against protozoan than macrophage cells. Transmission electron microscopy revealed many ultrastructure alterations, indicating damage and significant changes in the mitochondria of the parasite caused by eupomatenoid-5 [56]. Some is known about parasite mitochondria damage, cytoplasmic vacuolization and the presence of myelin-like figures caused by some drugs, including eupomatenoid-5. Rodrigues and co-workers (2002) [57] suggested that some drugs can inhibit the protozoa sterols biosynthesis and this can produce ergosterol depletion that can alter the physical properties of the membrane. Thus, the tested neolignans **4**, **5** and **6**, structurally related to eupomatenoid-5 (**3**), can act in the sterols biosynthesis producing mitochondria damage.

Among all tested compounds, conocarpan (**6**) showed the highest value (µg/mL) of 50% reduction in the number of viable cells for fibroblasts, macrophages and kidney epithelial cells (Table 3). This compound also showed the highest SI in all three mammalian cells for *Candida* and *Leishmania* strains (Tables 4 and 5), pointing out this compound as the less cytotoxic of the tested.

Table 1. Antimicrobial activity (*Candida* spp.) of extracts and neolignans from leaves of *Piper rivinoides* Kunth (Piperaceae).

Material	<i>C. albicans</i> PRI		<i>C. albicans</i> ATCC 10231	
	MIC (mM)	IC ₅₀ (mM)	MIC (mM)	IC ₅₀ (mM)
Hydroalcoholic extract	91.80±16.80	46.60±5.70	122.20±15.00	68.40±7.90
Hexane fraction	17.80±5.54	8.0±1.1	28.7±9.7	16.0±4.8
Dichloromethane fraction	236.90±95.60	129.10±48.50	191.40±29.70	58.50±14.70
Ethyl acetate fraction	NA	NA	NA	NA
<i>n</i> -Butanol fraction	NA	NA	NA	NA
Eupomatenoid-5 (3)	NA	NA	NA	NA
Compound (4)	230.10 ^a ±48.00 (0.7774±0.1622)	111.10±38.80 (0.3753±0.1311)	232.50 ^a ±10.60 (0.7855±0.0356)	113.40±4.80 (0.3831±0.0162)
Eupomatenoid-6 (5)	140.40 ^b ±25.60 (0.5312±0.0968)	75.60±22.10 (0.2860±0.0836)	118.50 ^b ±3.50 (0.4483±0.0132)	65.10±3.80 (0.2463±0.0143)
Conocarpan (6)	20.50 ^c ±0.70 (0.0770±0.0026)	9.20±1.20 (0.0345±0.0045)	39.40 ^c ±1.40 (0.1479±0.0053)	19.60±2.30 (0.0736±0.0086)
Fluconazole	> 250.00 ^d	NC	3.76 ^d ±0.22 (0.0123±0.0007)	1.72±0.11 (0.0056±0.0003)

The Minimum Inhibitory Concentrations and Inhibitory Concentration of 50% (MIC and IC₅₀, respectively) are expressed in µg/mL. Milimolar (mM) values are expressed in parentheses. NA= No activity. NC = not calculated. Compound (4) = (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(E)-propenylbenzofuran. Values with the same letter in the same column don't differ statically.

Table 2. Antiparasitic activity (*Leishmania* spp.) of extracts and neolignans from leaves of *Piperrivinoides* Kunth (Piperaceae).

Material	<i>L. amazonensis</i>		<i>L. chagasi</i>	
	MIC (mM)	IC ₅₀ (mM)	MIC (mM)	IC ₅₀ (mM)
Hydroalcoholic extract	> 500	NC	281.60 ± 0.04	140.04 ± 0.04
Hexane fraction	> 500	NC	> 500	NC
Dichloromethane fraction	> 500	NC	> 500	NC
Ethyl acetate fraction	> 500	NC	> 500	NC
<i>n</i> -Butanol fraction	> 500	NC	> 500	NC
Eupomatenoid-5 (3)	376.10 ^a ± 0.04 (1.2777±0.0010)	126.10 ± 0.04 (0.4284±0.0001)	189.00 ^a ±0.07 (0.6421±0.0002)	75.50±0.07 (0.2565±0.0002)
Compound (4)	79.50 ^b ±0.07 (0.2686±0.0002)	29.00 ^a ±0.07 (0.0980±0.0002)	134.50 ^b ±0.10 (0.4544±0.0003)	53.00±0.10 (0.1791±0.0003)
Eupomatenoid-6 (5)	100.00 ^c ±0.06 (0.3783±0.0002)	39.50±0.06 (0.1494±0.0002)	110.00 ^c ±0.10 (0.4162±0.0030)	38.00±0.10 (0.1438±0.0030)
Conocarpan (6)	80.00 ^b ±0.04 (0.3004±0.0002)	10.80 ^b ±0.04 (0.0405±0.0002)	94.00 ^d ±0.10 (0.3529±0.0004)	24.50±0.01 (0.0920±0.0004)

The Minimum Inhibitory Concentrations and Inhibitory Concentration of 50% (MIC and IC₅₀, respectively) are expressed in µg/mL. Milimolar (mM) values are expressed in parentheses. NA= No activity. NC = not calculated. Compound (4) = (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(E)-propenylbenzofuran. Values with the same letter in the same column don't differ statically.

Table 3. Results of 50% reduction in the number of viable cells (CC_{50}) of the pure compounds on lines of fibroblasts cells (L929), macrophages (RAW) and kidney epithelial cells (VERO).

Cells	Eupomatenoid-5 (3)	Compound (4)	Eupomatenoid-6 (5)	Conocarpan (6)
L929	11.0 ^a	19.0 ^b	8.6 ^c	38.0 ^d
RAW	6.0 ^a	9.6 ^b	4.6 ^c	11.3 ^d
VERO	8.9 ^a	11.3 ^b	4.4 ^c	8.4 ^a

CC_{50} values are expressed in $\mu\text{g}/\text{mL}$. Compound (4) = (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(E)-propenylbenzofuran. Values with the same letter in the same line don't differ statically.

Table 4. Neolignans selectivity index (SI) for *Candida* strains in fibroblasts cells (L929), macrophages (RAW) and kidney epithelial cells (VERO).

Compound	<i>C. albicans</i> PRI			<i>C. albicans</i> ATCC 10231		
	L929	Raw	VERO	L929	Raw	VERO
Eupomatenoid-5 (3)	---	---	---	---	---	---
Compound (4)	0.17	0.09	0.10	0.17	0.08	0.01
Eupomatenoid-6 (5)	0.11	0.06	0.06	0.13	0.07	0.07
Conocarpan (6)	4.13	1.23	0.91	1.94	0.58	0.43

SI = selectivity index = CC_{50}/IC_{50} ; compound (4) = (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(E)-propenylbenzofuran.

Table 5. Neolignans selectivity index (SI) for *Leishmania* strains in fibroblasts cells (L929), macrophages (RAW) and kidney epithelial cells (VERO).

Compound	<i>L. amazonensis</i>			<i>L. chagasi</i>		
	L929	Raw	VERO	L929	Raw	VERO
Eupomatenoid-5 (3)	0.09	0.05	0.07	0.15	0.08	0.12
Compound (4)	0.66	0.33	0.39	0.36	0.18	0.21
Eupomatenoid-6 (5)	0.22	0.12	0.11	0.23	0.12	0.12
Conocarpan (6)	3.52	1.05	0.78	1.55	0.46	0.34

SI = selectivity index = CC_{50}/IC_{50} ; compound (4) = (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(E)-propenylbenzofuran.

3.3. Quantitative approach

The *n*-hexane fraction showed the highest antimicrobial activity on the yeast *Candida albicans* and *Leishmania chagasi* (Tables 1 and 2). This result suggests a synergistic relation of the lignans present in the sample. Thus, we evaluated the neolignans content in the bioactive fraction using NMR data. Since we could not validate the methodology, the discussion bellow can suggest an estimative of neolignans in the bioactive fraction. A fundamental feature of NMR spectroscopy is that the intensity of a single resonance obtained will have direct relation with the number of spins and so with the concentration of that compound. So, if the NMR experiment is performed in a precise way that each intensity interfering parameter is optimized one can correlate directly the integrated signal to the concentration of a certain compound [42]. Considering this, key known resonances observed for eupomatenoid-5 (**3**) (^1H , d, $^4J_{\text{HH}}=1.6$ Hz, δ 7.37 ppm; H-6), eupomatenoid-6 (**5**) (^1H , d, $^3J_{\text{HH}}=8.68$ Hz, δ 7.66 ppm; H-6' and H-2') and conocarpan (**6**) (^1H , d, $^2J_{\text{HH}}=8.10$ Hz, δ 6.76 ppm; H-3) were used to estimate the neolignans amount. The integrals of each of the nominated resonances above were used as their absolute values of integration for quantitative calculations. The results found with basis on the equation (1) (see experimental) showed that the *n*-hexane fraction **contains 29.8% of**

eupomatenoid-5 (**3**), **7.6%** of eupomatenoid-6 (**5**) and **5.5%** of conocarpan (**6**). The compound (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran (**4**) did not show any measurable selective proton resonance. Protons of 2-(4'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran (**1**) and eupomatenoid-3 (**2**) were not observable at the *n*-hexane fraction ¹H-NMR spectra for suitable quantification due their low percentage in the extract. Percentages were calculated for the total powdered and dry plant material and demonstrated a total of **42.9%** of neolignans (eupomatenoid-5 + eupomatenoid-6 + conocarpan) in the analyzed bioactive fraction. As it can be seen, the *n*-hexane fraction showed higher bioactivity even with minor concentrations of conocarpan (5.5%) that was characterized as the most active tested neolignan. Markedly, more studies must be employed to elucidate the mode of action of those purified compounds and the synergistic relation between them.

4. Conclusion

Our findings stand out *Piper rivinoides* as an important source of benzofuran and dihydrobenzofuran neolignans, natural compounds with many biological activities. Further mechanistic studies need to be done to achieve the structure-activity relationship that is responsible for the antifungal and antileishmanial activities of neolignans, but we are contributing on this field since we have demonstrated higher anticandidal and antileishmanial activity of a 4'-hydroxy-2,3-dihydrobenzofuran neolignan (conocarpan) and that a free hydroxyl group at position 4' is very important to increase the antimicrobial activity of benzofuran or dihydrobenzofuran neolignans. Eupomatenoid-5 (**3**), (2R,3R)-2,3-dihydro-2-(4'-hydroxy-3'-methoxyphenyl)-3-methyl-5(*E*)-propenylbenzofuran (**4**), eupomatenoid-6 (**5**) and conocarpan (**6**) can be considered toxic for mammalian cells, in accordance with our findings in terms of SI. However, the activity of these compounds against *Candida* and *Leishmania*, here demonstrated, justify further studies with semi-synthetic benzofuran or dihydrobenzofuran neolignans in order to improve the observed antimicrobial activities and to reduce the toxicity.

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