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Anti-Mycobacterium tuberculosis Activity of Compounds from *Cedrela fissilis* Vell. Seeds (Meliaceae)

Atividade anti-Mycobacterium tuberculosis de compostos das sementes de Cedrela fissilis Vell. (Meliaceae)

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The phytochemistry of the species *Cedrela fissilis* Vell., family Meliaceae, also popularly known as "cedro rosa", was investigated, leading to the isolation of four compounds from the crude methanolic extract of its seeds; the triterpene Piscidinol A **(1)**, and three limonoids, Andirolide N **(2)**, Mexicanolide **(3)**, and Proceranolide **(4)**. Subsequently, the antimycobacterial, anti-inflammatory, and cytotoxicity activities of these compounds were investigated. The Proceranolide limonoid **(4)** presented the best result for the investigated biological activities, with moderate antimycobacterial activity, considerable inhibition of nitric oxide production, and low cytotoxicity. The Andirolide N **(2)** and Proceranolide **(4)** limonoids are being reported for the first time in the *Cedrela* genus.

Keywords: *Cedrela fissilis*; Meliaceae; limonoids; biological activities

1. Introduction

Tuberculosis (TB) is a bacterial infectious disease[1], neglected, with high mortality rates in the world.[2] Transmission occurs via the respiratory tract, which primarily affects the lungs. However, other organs can be affected.[3] TB infection may be present in latent or active forms, whereby in the first there are no symptoms and in the second, symptoms include fever, dry or productive cough, sore throat, sweating, weight loss, and tiredness or weakness.[3]

According to the WHO, 484,000 people developed resistance to rifampicin in 2019, which is the most active medicine against tuberculosis bacillus, with 78% of these being multiresistant.[2] Therefore, there is a necessity to develop new forms of diagnosis and treatment.

Brazil stands out in terms of strategies to contain tuberculosis, having achieved an 8% reduction in the number of deaths from tuberculosis in the last decade.[4] In addition, new forms of treatment and diagnosis were incorporated into the Single Health System (SUS) in 2020.[5] It is worth mentioning that the SUS offers, free of charge, diagnosis, treatment, and follow-up by specialized professionals.[7] Nonetheless, actions to contain the incidence of cases, especially among the population deprived of liberty (PDL), need to be intensified. The Report of Tuberculosis in Brazil outlined 73,864 cases in 2019, of which 11.1% were among PDL, surpassing the cases of co-infection with HIV.[8]

Research into secondary metabolites of Meliaceae species and their biological activities have increased, as these plants present activities such as insecticidal, antiviral, anthelmintic, anti-rheumatic, anti-cancer, and anti-inflammatory, which are usually related to modified triterpenes, denominated limonoids.[9] These activities have also been described for the *Cedrela* species.[9] The *Cedrela* genus contains 18 species[10,11] and the majority of the chemical compounds described in the literature for this species are terpenoids. In addition, 54.6% of the triterpenoids found are limonoids.[12]

The current study aimed to isolate secondary metabolites from crude methanolic extract of *Cedrela fissilis* seeds and evaluate their antimycobacterial, anti-inflammatory, and cytotoxic activities

2. Results and Discussion

The triterpene, Piscidinol A (**1**)[13], and three limonoids, Andirolide N (**2**)[14], Mexicanolide (**3**)[15], and Proceranolide (**4**)[16] (Figure 1), were isolated from the methanolic extract of *C.*

fissilis seeds, characterized by their spectral data of ^{1}H and ^{13}C-NMR (1D and 2D) and high resolution spectrometry (HRESIMS), and compared with data described in the literature (supplementary material).

These limonoids (**2**-**4**) were classified as mexicanolide type, and are reported as the main type of limonoid of the genus *Cedrela*.[12] In the comparison of their structures (Figure 1), the main differences observed are restricted to the carbon atom C-3 (sp^3 oxygenated, **2** and **4**, or carbonyl sp^2, **3**) and the double bond positions (Δ^{8-14}, **3** and **4**, or D^{14-15}, **2**).

The antimycobaterial, anti-inflammatory, and cytotoxicity activities of these compounds were investigated and the corresponding minimum inhibitory concentration 50% (MIC_{50}), shown in Table 1.

All compounds were active with antimycobacterial activity against both strains of *M. tuberculosis*. However, compared to rifampicin MIC_{50} values, proceranolide (**4**) showed moderate inhibitory effects, with MIC_{50} values for H37Rv and M299 of 37.6 ± 0.4 µg/mL, and 44.9 ± 1.0 µg/mL, respectively, while mexicanolide (**3**) showed weak inhibitory effects ($MIC_{50\text{-}H37Rv}$ = ≥500 µg/mL; $MIC_{50\text{-}M299}$ = 347.7 ± 1.0 µg/mL). The effects of these compounds on the growth of both strains of *M. tuberculosis* are shown in Figure 2.

In the case of inhibition of nitric oxide (NO) production, all compounds showed some activity. However, limonoid **4** (26.9 ± 0.6 µg/mL) demonstrated considerable inhibition when its IC_{50} values were compared to the positive control (L-NMMA - 13.2 ± 0.6 µg/mL) (Table 1). The effects of each of the compounds on the inhibition of NO production are reported in Figure 3A.

These compounds were also evaluated for their cytotoxicity, which was observed in all compounds tested (Table 1). However, **4** showed higher IC_{50} values than the others. Figure 3B presents the cytotoxicity effects.

Boeno *et al.*[17] carried out a biological assay with similar methodology and evaluated the anti-mycobacterial

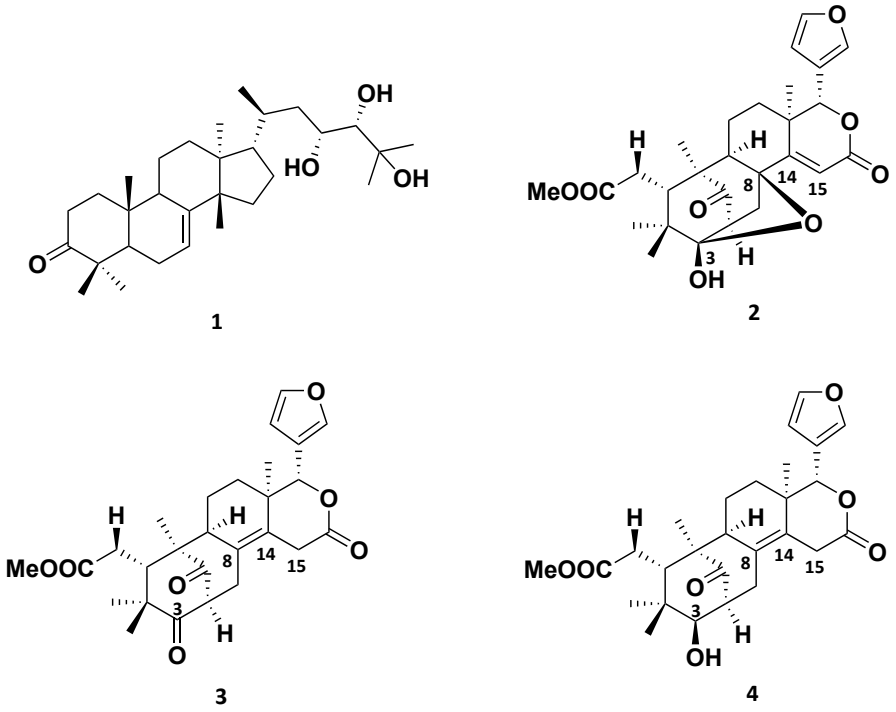

Figure 1. Compounds isolated from C. fissilis seeds

Table 1. Inhibitory effects of the compounds isolated from *C. fissilis* seeds on growth of *M. tuberculosis* H37Rv and M299 strains in culture, NO production by LPS-stimulated RAW 264.7 macrophages, and assessment of cytotoxicity

Compounds	MIC_{50} (µg/mL)		IC_{50} (µg/mL)	
	H37Rv	M299	NO	MTT
1	49.6 ± 0.5	66.0 ± 1.0	36.2 ± 0.7	47.4 ± 0.2
2	60.1± 0.3	50.4 ± 1.0	43.9 ± 0.9	225.9 ± 0.2
3	≥500	347.7 ± 1.0	30.6 ± 0.7	≥500
4	37.6 ± 0.4	44.9 ± 1.0	26.9 ± 0.6	321.3 ± 0.1
Rifampicin[1]	0.8 ± 0.1	0.9 ± 0.2	XX	XX
L-NMMA[2]	XX	XX	13.2 ± 0.6	XX

[1] Standard antimycobacterial drug; [2] Standard nitric oxide inhibitor; Mean value ± SD; XX - not defined

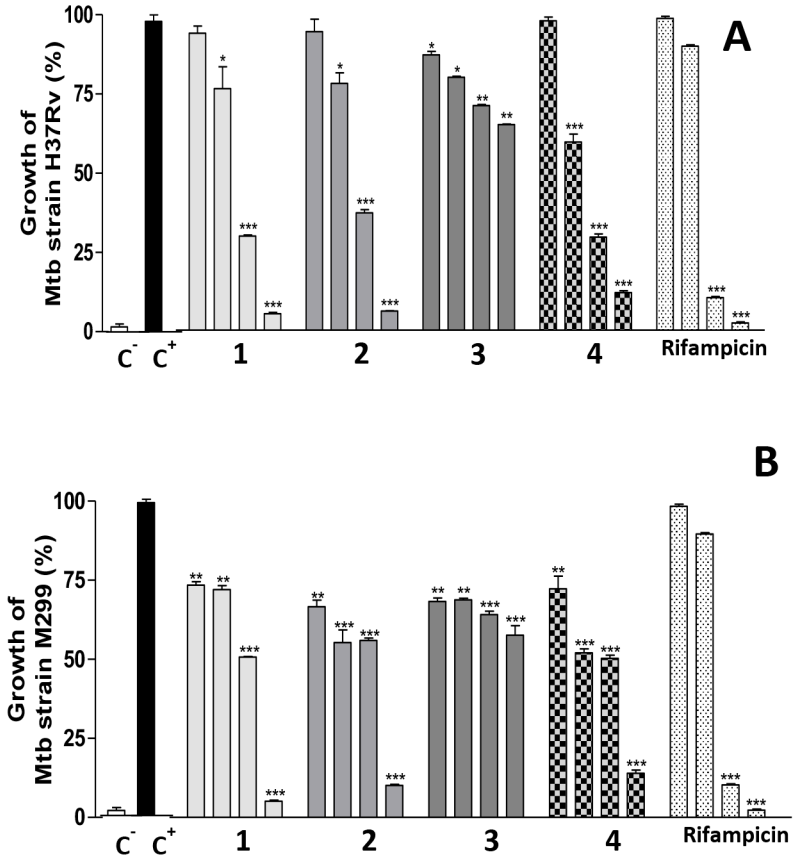

Figure 2. Effects of the compounds from *C. fissilis* on M. tuberculosis growth in bacterial culture. Bacterial suspensions (1×10^6 CFU/well) of M. tuberculosis strain H37Rv (A) and clinical isolate M299 (B) were treated or untreated with samples (4, 20, 100 and 500 µg/mL) or rifampicin (0.00032, 0.0016, 0.008, 1 µg/mL for the strain H37Rv and 0.008, 0.04, 0.2 and 10 µg/mL for the strain M299) for 5 days. Bacterial growth in the resulted cultures was quantified by MTT test. Data are presented as a percentage of bacterial growth of each treated culture compared to the growth of corresponding untreated culture (100%). The four bars for each compound refer to concentrations tested in ascending order. Bacterial suspensions treated with antibiotic rifampicin and culture medium 7H9 supplemented with ADC (C-) were used as a negative control. Untreated bacterial suspension served as a positive control (C+). The results presented are mean values obtained over three experiments, each done in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared to untreated group

activity and cytotoxicity of triterpenes, and another class of compounds isolated from *Homalolepis suffruticosa* Engl. (Simaroubaceae).

The results for Piscidinol A (**1**) were consistent with those presented by Boeno *et al.*[17] for Hispidol A, Nilocitine, and α-dihydronylocytine; all of these triterpenes are tirulan type. Thus, it can be deduced that the OH group at C-3 contributes to inhibition potential of *Mycobacterium* growth, for both strains of *M. tuberculosis*. Hispidol showed better MIC values than Piscidinol (**1**), and similar to the rifampicin MIC_{50}. On the other hand, all of these triterpenes were cytotoxic.

With respect to the limonoids, Passos *et al.*[18], using a similar methodology to that described in the current paper, tested the anti-tuberculosis, anti-inflammatory, and cytotoxic activities of the limonoids Morenolide, Nimbinene, Nimbinal, Nimbandiol, and Salannin, and also compounds of other classes, which were isolated of *Azadirachta indica* roots. These limonoids are classified as C-*seco*, and are more active and cytotoxic than limonoids **2**-**4**, which are B,D-*seco*.

It is suggested that the structural abundance of limonoids should be studied, and possible structural modifications carried out to verify the possibility of more active or less cytotoxic compounds.

3. Material and Methods

3.1. General experimental procedures

Column Chromatography (CC) was performed on silica gel 60 (0.063-0.200 mm, Merck). Preparative Thin Layer Chromatography (PTLC) was performed on silica gel 60 PF_{254} containing gypsum (Merck). Methanol (CH_3OH - 99.8%), dichloromethane (CH_2Cl_2 - 99.5%), *n*-hexane (98.5%), acetone (99.5%), and ethyl acetate (EtOAc - 99.5%) were used as mobile phase solvents, purchased from Synth (São Paulo, Brazil). 1D and 2D NMR experiments were performed using a 500 MHz Bruker Ascend 500 NMR spectrometer, operating at 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR. Deuterated solvents, chloroform

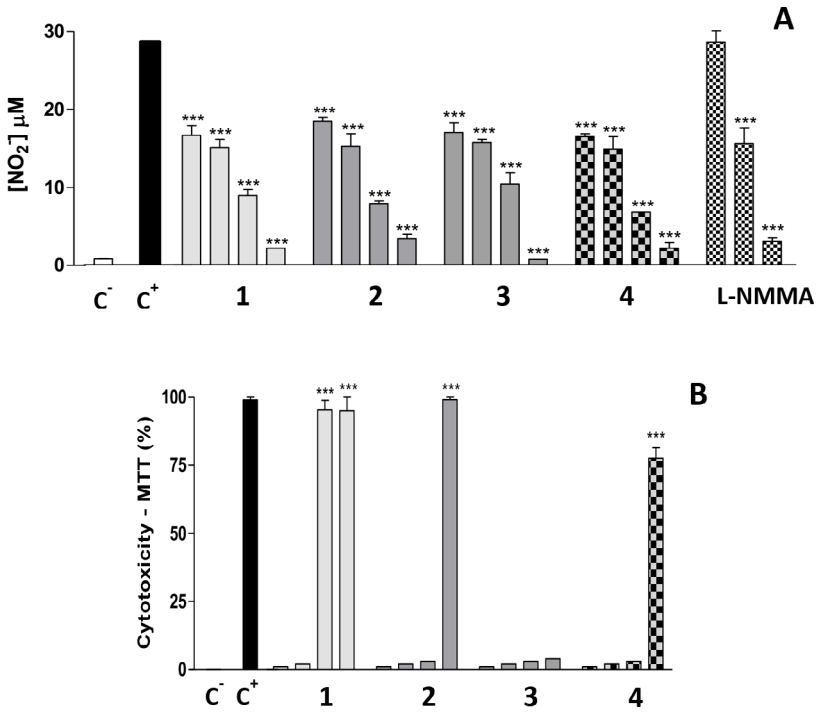

Figure 3. Inhibitory effect of the compounds on NO production in macrophages RAW 264.7 stimulated by LPS (A) and cell citotoxicity evaluation by MTT assay (B). Plated RAW 264.7 macrophages ($5x10^5$ cells / mL) were stimulated by LPS (1 µg / mL) and treated with the recommendations of 4, 20, 100 and 500 µg/mL for 24 h. Evaluation of NO production took place using the Griess method. As negative control of NO production (C-), culture of unstimulated and untreated macrophages was used and as positive control (C +), culture of macrophages only stimulated by LPS. L-NMMA was used at the concentrations 4, 20 and 100 µg/mL(A). In the toxicity tests, after 24 h of the stimulated and treated cell culture, 10µL of the MTT solution / well was added and the formazan crystals solubilized in acidified isopropanol. As a negative control (C-) of cell death, a culture of macrophages only stimulated by LPS was used and as a positive control (C +), a culture of macrophages treated with 1% Triton X-100 (B). Values were represented as mean ± standard deviation and different groups considered significant according to $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

($CDCl_3$), methanol (CD_3OD), and pyridine (pyridine-d_5), containing TMS (tetramethylsilane) as an internal standard, were used. HR-ESI-MS were obtained on a micrOTOF-Q II Bruker Daltonics mass spectrometer, with the use of the positive ion mode of analysis.

3.2. Plant material

Cedrela fissilis (SisGen code AC8E4F3) seeds were collected in July 2018 at Campos dos Goytacazes, RJ, Brazil (latitude 21°41'56.9" S, longitude 41°12'22.0" W). The voucher specimen (H11234) was deposited in the herbarium of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF).

3.3. Extraction and isolation

Cedrela fissilis seeds were dried and powdered. The extraction was performed with methanol (CFM, 49.4 g). Part of the methanolic extract (24.3 g) was fractionated by silica gel CC, with a polar gradient of CH_2Cl_2:MeOH, obtaining 8 fractions (CFM1 - CFM9). Mexicanolide (**3**- 138.9 mg) was isolated as precipitate of fraction CFM5. CFM6 was chromatographed, with a polar gradient of *n*-hexane: acetone, generating 11 fractions (CM6.1 - CM6.11). Fraction CFM6.7 (764.2 mg) was chromatographed, with a polar gradient of *n*-hexane: EtOAc, obtaining 11 fractions (CFM6.7.1 - CFM6.7.11). Proceranolide (**4**) was identified in fraction CFM6.7.8 (286.9 mg). Fraction CFM6.7.7 was chromatographed, with a polar gradient of *n*-hexane: EtOAc, obtaining 4 fractions (CFM6.7.7.1 - CFM6.7.7.4). PTLC was performed with fraction CFM6.7.7.1 (44.4 mg), with 2% of CH_2Cl_2:MeOH, obtaining piscidinol A (**1**- 11.7 mg) and andirolide N (**2**- 10.5 mg) of fractions CFM6.7.7.1.A and CFM6.7.7.1.B, respectively.

3.4. Antimycobacterial activity assay

Two *Mycobacterium tuberculosis* strains (low virulent laboratory strain H37Rv, ATCC 27294, and highly virulent *Mycobacterium tuberculosis* Beijing strain M299 isolated from TB patient in Mozambique) were evaluated for virulence in previous study.[19] Mycobacterial strains were grown in suspension in 7H9 Middlebrook medium, containing 10% albumin dextrose complex (ADC), 0.5% glycerol and 0.05% Tween-80 at 37 °C, under Biosecurity level 3 containment conditions. The bacterial suspensions were plated (1 × 106 CFU/well in 96-well plate) and incubated in the presence of compounds at concentrations of (4, 20, 100, and 500 µg/mL) or rifampicin (ranging from 0.00032 to 1 µg/mL for *M. tuberculosis* H37Rv strain and from 0.008 to 10 µg/mL for clinical *M. tuberculosis*

isolate M299). The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay was performed to evaluate the bacterial growth[20], using the procedures described by Ventura *et al*.[21]

Both strains of *Mycobacteria* were incubated with Middlebrook 7H9 medium supplemented with 0.05% glycerol, 0.05% Tween 80, and ADC (albumin dextrose catalase). Cells were plated at a density of 1 × 106 CFU/well in a 96-well plate and treated with the samples. The plate was incubated at 37 °C and 5% CO_2 for 5 days. Subsequently, the MTT solution was incubated for 3 h, and the lysis buffer was added [20% w/v sodium dodecyl sulphate (SDS) and 50% dimethylformamide (DMF) in distilled water, pH 4.7]. The plate was incubated overnight, and the reading was carried out using a spectrophotometer at 570 nm. As a positive control was used untreated *M. tuberculosis* and negative control was used Middlebrook 7H9 medium.

3.5. Anti-inflammatory activity assay

Macrophages RAW 264.7 were obtained from the American Type Culture Collection (ATCC) and grown at 37 °C and 5% CO_2 in DMEM F-12 supplemented with 10% FCS and gentamicin (50 µg/mL). Posteriorly, they were seeded in 96-well tissue culture plates in the presence or absence of four concentrations of the samples (4, 20, 100, and 500 µg/mL) and/or LPS (Escherichia coli 055:B5; Sigma-Aldrich) for 24h of incubation. Afterward, supernatants were collected, and the concentration of nitric oxide was determined by the Griess Method.[22] As a positive control, NG-methyl-L-arginine acetate salt (L-NMMA, Sigma-Aldrich, 98% purity) was used at concentrations of 4, 20 and 100 µg/mL.

3.6. Cytotoxicity assay

Cytotoxicity was evaluated with the MTT assay, checking the mitochondrial-respiration-dependent reduction.[23] Macrophages RAW264.7 (5 × 105 cells/mL) were incubated at 37 °C in 5% CO_2 for 24 h in 96-well plates with increasing doses of the test compound (4.0, 20, 100, and 500 µg/mL). Posteriorly, 5 µL of MTT solution (5 mg/mL) were added to each well. After incubation for 2 h at 37 °C, the formazan crystals in viable cells were solubilized in HCl (4 mM) and added to isopropanol. The absorbance of each well was then read at 570 nm. Non-treated cells were used as a positive control and 1% Triton X-100 detergent-treated cells as a negative control.

3.7. Statistical analysis

Results were tabulated by LabChart 7 and statistically analyzed using GraphPad Prisma 4. The tests were performed in triplicate and the values are expressed as mean ± SD.

4. Conclusions

Four compounds were isolated from the seeds of *Cedrela fissilis*, namely: Piscidinol A (**1**), Andirolide N (**2**), Mexicanolide (**3**), and Proceranolide (**4**). Compunds **2** and **4** are being reported for the first time in *Cedrela* genus. Moreover, compound **4** presented moderate antimycobacterial activity and a considerable inhibitory effect on NO production and low cytotoxicity.

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