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Chemical Constituents from Branches of *Maytenus gonoclada* (Celastraceae) and Evaluation of Antimicrobial Activity

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Seis triterpenos pentacíclicos isolados dos galhos de *Maytenus gonoclada* (Celastraceae), incluindo todos os dados de RMN do novo composto 3-oxo-12α,29-diidroxifriedelano são aqui relatados. A estereoquímica do novo friedelano foi estabelecida por dados de RMN bidimensional (HSQC, HMBC e NOESY), e sua massa molecular confirmada por espectrometria de massas (ESI). Testes de atividade antimicrobiana usando método de difusão em disco e de macrodiluição foram realizados contra as bactérias *Escherichia coli*, *Citrobacter freundii* e *Bacillus cereus*, e contra o fungo *Candida albicans*. O triterpeno 3-oxo-12α-hidroxifriedelano mostrou resultado positivo contra *C. albicans*.

Six pentacyclic triterpenes were isolated from branches of *Maytenus gonoclada* (Celastraceae) and all NMR data of a new compound 3-oxo-12α,29-dihydroxyfriedelane are herein reported. The stereochemistry of the new friedelane was established by bidimensional NMR (HSQC, HMBC and NOESY) data, and its molecular weight confirmed by ESI mass spectrometry. Antimicrobial activity assays using the method of disk diffusion and macrodilution were carried out against the bacteria *Escherichia coli*, *Citrobacter freundii*, and *Bacillus cereus*, and against the fungi *Candida albicans*. The triterpene 3-oxo-12α-hydroxyfriedelane showed positive result against *C. albicans*.

Keywords: *Maytenus gonoclada*, Celastraceae, 3-oxo-12α-hydroxyfriedelane, 3-oxo-12α,29-dihydroxyfriedelane, antimicrobial activity

Introduction

Celastraceae family contains many species that have been extensively studied in function of their use in traditional medicine. Some species of *Maytenus* genus are worldwide distributed and have been used by Africans to treat cancer, by Asian people as an insecticide,[1] and by the South American people on the treatment of gastrointestinal diseases.[2] The biological activities associated to *Maytenus* species have been assigned to different classes of secondary metabolites such as phenolic glucosides,[3] flavonoids[4] and triterpenes.[5]

Pentacyclic triterpenes (PCTT) have been commonly isolated from species of the Celastraceae family, and some of them, like 3-oxofriedelane and 3β-hydroxyfriedelane, are considered taxonomic markers of *Maytenus* genus.[4] A large number of pharmacological activities has been associated to triterpenes isolated from species of the *Maytenus* genus, such as, 3,15-dioxo-21α-hydroxyfriedelane, isolated from *Maytenus robusta*, that showed antiulcerogenic activity,[6] and maytenfolic acid, isolated from *Maytenus heterophylla*, that showed growth inhibitory effect on *Candida albicans*.[7]

Maytenus gonoclada Martius, popularly known as "tiuzinho", can be found in regions of "cerrado" and rupestrian fields of Southeastern and Northeastern Brazil. In our previous studies of the hexane extract from *M. gonoclada* leaves, the occurrence of five PCTT of the friedelane series was reported.[8]

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The present paper reports the phytochemical study of hexane extract from branches of *M. gonoclada*, which was isolated five known triterpenes: 3-oxofriedelane (**1**), 3β-hydroxyfriedelane (**2**), 3-oxo-12α-hydroxyfriedelane (**3**), 3,11-dioxofriedelane (**4**) and 3,16-dioxofriedelane (**5**).[8] In addition, a new compound of the friedelane series, 3-oxo-12α,29-dihydroxyfriedelane (**6**), was also isolated and its structure was developed by detailed ^{1}H and ^{13}C NMR analysis, including bidimensional (HSQC, HMBC and NOESY) spectral data.

The NMR data of compound **6**, as well as the complete hydrogen chemical shifts assigned to compound **5**, that have not been described in the literature yet, are herein reported for the first time.

An evaluation of the antimicrobial activity of the hexane extract is also reported here. The compounds 3-oxofriedelane (**1**), 3-oxo-12α-hydroxyfriedelane (**3**), 3,16-dioxofriedelane (**5**) and 3-oxo-12α,29-dihydroxyfriedelane (**6**) were screened by the methods of disk diffusion and macrodilution. The assays were based on the growth inhibition of standard strains of *Escherichia coli*, *Citrobacter freundii*, *Bacillus cereus*, and the yeast *Candida albicans*. Minimal inhibitory concentration (MIC), produced by hexane extract and 3-oxo-12α-hydroxyfriedelane (**3**) were determined. The results showed that 3-oxo-12α-hydroxyfriedelane (**3**) presented growth inhibition activity.

Experimental

General experimental procedures

Column chromatography (CC) was carried out using silica gel 60 (70-230 Mesh, Merck) and for thin layer chromatography (TLC) it was employed precoated silica gel plates. The detection of spots was made by spraying a mixture (1:1) of vanillin (ethanol solution, 1% m/v) and perchloric acid (aqueous solution, 3% v/v).[9] A Mettler FP 80 HT apparatus was used to determine melting points (uncorrected). Elemental analyses were performed on a CHN Perkin-Elmer 2400 apparatus. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter using a 100 mm, 1.0 mL cell tube capacity. Infrared spectra were recorded on a Perkin Elmer, Spectrum One spectrophotometer (ATR). Mass spectrometry was conducted in a LCQFleet (Thermo Scientific, San Jose, CA) bearing an electrospray ionization (ESI) source, operating in the positive mode. ESI source conditions were: heated capillary temperature of 290 °C, sheath gas (N_2) flow rate at 20 (arbitrary units), spray voltage of 4.8 kV, and capillary voltage of 2.0 V.

The ^{1}H and ^{13}C NMR spectra were measured on a Bruker DRX 400 Avance spectrometer at 400 and 100 MHz at 300 K, equipped with inverse detection 5 mm multinuclear head $^{1}H/^{13}C$. Each compound was dissolved in $CDCl_3$ or in $CDCl_3$ with 2 drops of pyridine-d_5, and transferred to a 5 mm o.d. NMR tube. TMS was used as internal standard ($\delta_H = \delta_C = 0$). Bi-dimensional (2D) NMR spectra were acquired under standard conditions. Data processing was carried out on SGI workstation using the Bruker (DRX 400) software.

Plant material

Samples of *M. gonoclada*'s branches were collected in Serra da Piedade, Caeté, Minas Gerais, Brazil, in October 2004. Botanical identification was provided by Dr. Rita Maria Carvalho-Okano. The voucher specimen (HBCB 60280) was deposited in the Herbarium of the Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

Extraction and isolation

The branches of *M. gonoclada* were dried at room temperature, milled and the resulting powder (1000 g) was submitted to extraction with hexane in a Soxhlet apparatus. The formation of a white solid material was observed during the removal of the hexane in a rotatory evaporator. This solid was separated by filtration; the dried material (1.0 g) was submitted to silica gel (50 g) CC eluted with hexane, chloroform, ethyl acetate and ethanol in mixtures of increasing polarity. Fifty-seven fractions of 200 mL each were obtained and grouped according to the similar profiles observed in the chromatoplates. Fractions 22 to 37 were eluted with hexane-chloroform (1:1). After solvent evaporation, fraction (Fr.) 22 produced a white solid (5.0 mg), which was identified as 3-oxofriedelane (**1**). Fractions 23-27 provided a white solid (112.2 mg), which was rechromatographed furnishing additional amount (35.0 mg) of 3-oxofriedelane (**1**) and 3β-hydroxyfriedelane (**2**) (15.2 mg). These PCTTs were characterized by their respective ^{13}C NMR spectral data, being also compared to published data. Fractions 34-35 gave a white solid (34.4 mg) that was identified by NMR spectra analyses and comparison with published data as a mixture of 3,11-dioxofriedelane (**4**) and 3,16-dioxofriedelane (**5**). The group of fractions Fr.36-37 (234.0 mg) was determined as a mixture, by TLC, and named as material A. The group Fr.38-43 (173.2 mg) (eluted from hexane/chloroform 3:7) was submitted to flash CC (silica gel 230-400 Mesh, Merck, 5.8 g) eluted with hexane, chloroform, ethyl

acetate and methanol in mixtures of increasing polarity. Forty-seven fractions of 10 mL each were obtained yielding the 3-oxo-12α-hydroxyfriedelane (**3**) (10.3 mg) and 3,16-dioxofriedelane (**5**) (12.0 mg). Using TLC, the fraction 56 (56.2 mg) (eluted with ethyl acetate) was also characterized as a mixture, and then named as material B.

Material A (234.0 mg) was submitted to silica gel CC (10 g) eluted with pure hexane, chloroform and ethyl acetate, or in mixtures of increasing polarities, providing 73 fractions of 25 mL each. After solvent removal, Fr.A25 (eluted from hexane/chloroform 7:3) gave a white solid (14.1 mg) which was identified as 3,11-dioxofriedelane (**4**), and Fr.A27 (eluted from hexane/chloroform 7:3) (10.0 mg) was identified as 3,16-dioxofriedelane (**5**). The group of fractions A30-45 eluted from hexane/chloroform 1:1 was purified by recrystallization (ethanol with drops of acetone) producing a crystalline solid (32.6 mg), which was identified as 3-oxo-12α-hydroxyfriedelane (**3**).

Material B (56.2 mg) was submitted to silica gel CC (22.6 g) eluted initially with chloroform/ethyl acetate (1:1) and then with pure ethyl acetate, furnishing 75 fractions of 10 mL each. After solvent evaporation, Fr.B49-69 gave an amorphous solid (9.0 mg) that was identified as 3-oxo-12α,29-dihydroxyfriedelane (**6**).

*3,16-Dioxofriedelane (**5**)*

Amorphous white solid, mp 218-220 °C. NMR spectral data: See Table 1.

*3-Oxo-12α,29-dihydroxyfriedelane (**6**)*

Amorphous white solid, $[\alpha]_D^{20} = -23$ ($CHCl_3$), mp 250-254 °C. IR (ATR) ν_{max}/cm^{-1}: 3327, 2920-2850, 1712, 1455, 1389, 1294, 1245, 1218, 1204, 1170, 111, 1056, 1039, 999, 977, 918, 823 and 751. NMR spectral data: See Table 1. MS ((+)-ESI): *m/z* 423.35 (24%) [M + H – $2H_2O$]; 405.33 (100%).

Antimicrobial bioassays

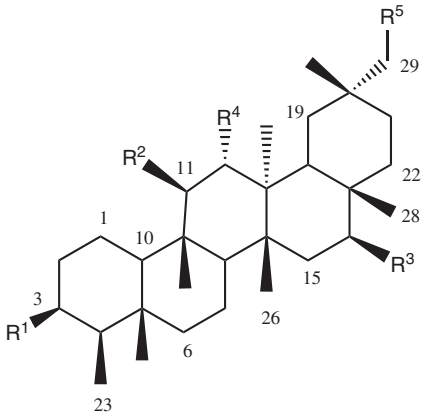
The bacteria strains, *E. coli* ATCC 25723, *B. cereus* ATCC 11778, *C. freundii* ATCC 29935 and the yeast *C. albicans* ATCC 18804 used in this study were obtained from American Type Culture Collection. The media broth heart infusion (BHI) was purchased from Merck (Darmstadt, Germany) and Biobrás (Montes Claros, Brazil). The strains were maintained on BHI medium and refrigerated at 7 °C.

Antimicrobial activity was evaluated using the disk diffusion method, according to the literature.[10] The microorganisms were cultivated in medium BHI and incubated for 18 h at 37 °C. Cells were suspended, according to the McFarland protocol, in saline solution to produce a suspension containing approximately 5×10^5 CFU mL^{-1}. An aliquot (10 μL) of this suspension was added to 10 mL of sterile antibiotic agar at 40 °C and then, inside a laminar flow cabinet, this mixture was poured onto an agar plate. Each tested compound (100 μg) was dissolved in chloroform and put on a paper disk (6 mm diameter), that was dried and placed on the agar plate. Each plate was constituted by 5 sample/disk, together with a disk containing chloramphenicol (30 μg) and another containing only chloroform that were used as positive and negative controls, respectively. The susceptibility of the bacteria and the yeast was determined by the formation of a growth inhibitory zone (mm) of each extract and tested compounds, observed after 18 h of incubation at 37 °C. Experiments were run in triplicate, and the results are presented as mean values of the three measurements. Chloramphenicol and miconazole were purchased from Sigma Chemical Co. (St. Louis, MO). The broth dilution test was used to evaluate the minimum inhibitory concentration (MIC) of growth and the initial inoculums contained 5×10^5 CFU mL^{-1}.[11] Tested compounds were dissolved in dimethyl sulfoxide (DMSO). Sequential dilutions provided the final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 μg/mL of tested compounds in BHI medium. Then, 100 μL of the inoculum was added to each tube. After an incubation time of 18 h at 37 °C, the lowest concentration of the tested compounds that inhibited the microorganisms growth (MIC) was visually determined. Tests using DMSO as negative control and chloramphenicol (for bacteria) and miconazole (for *Candida*) as positive controls were carried out in parallel. MIC tests were performed in duplicate with full agreement between both results.

Results and Discussion

A new triterpene (3-oxo-12α,29-dihydroxyfriedelane, **6**) and five known compounds (Figure 1) were isolated from the phytochemical study of the hexane extract of *M. gonoclada* branches. The known compounds were isolated in high degree of pureness and they were respectively identified as 3-oxofriedelane (**1**),[12,13] 3β-hydroxyfriedelane (**2**),[14] 3-oxo-12α-hydroxyfriedelane (**3**),[8] 3,11-dioxofriedelane (**4**),[15] 3,16-dioxofriedelane (**5**).[12,16] For the identification, their physical and spectral data were analyzed and the results compared with previously published data.

Compound **5** was isolated from hexane extract as a white amorphous solid, mp 218-220 °C, and showed positive Liebermann-Burchard (LB) test for triterpenes.[17] The carbon signals attributed to compound **5** were in accordance with the reported data.[12,16] Through 2D NMR



Compd.	R^1	R^2	R^3	R^4	R^5
1	=O	H	H	H	H
2	HO	H	H	H	H
3	=O	H	H	OH	H
4	=O	=O	H	H	H
5	=O	H	=O	H	H
6	=O	H	H	HO	HO

Figure 1. Chemical structures of the triterpenes isolated from branches of *M.gonoclada*.

spectral data (HSQC, HMBC and NOESY), the chemical shifts of all hydrogens assigned to compound **5** as well as its correlations were fully established (Figure 2 and Table 1). To the best of our knowledge, it is the first time that these data are reported.

Compound **6** (Figure 2) was obtained as an amorphous white solid, mp 250-254 °C, $[\alpha]_D^{20}$ −23, $CHCl_3$], and showed positive LB test for triterpenes.[17] The elemental analysis of compound **6** presented C 78.54% and H 10.97%, compatible with the molecular formula $C_{30}H_{50}O_3$ (MW 458 g mol^{-1}; calculated: C 78.55%; H 10.99%). ESI mass spectrometry confirmed this molecular weight, presenting a fragment *m/z* 423.35 (24%) [M + H - $2H_2O$]. This type of fragmentation is described for PCTT. According to Rhourri-Frih,[18] in PCTTs like betulin, the loss of two molecules of water occurs due to the presence of two hydroxyl groups, as observed for compound **6**. The IR spectra of **6** showed absorption bands: at 3327 cm^{-1}, compatible with the presence of hydroxyl group; at 2920 and 2850 cm^{-1}, characteristic of the alicyclic hydrocarbon; and also at 1712 cm^{-1}, which was attributed to a carbonyl group. The ^{13}C NMR and DEPT 135 spectra showed 30 signals: seven primary, eleven secondary, five tertiary and seven quaternary carbons, which were according to the PCTT skeleton. The ^1H and ^{13}C NMR data indicated the compound **6** as friedelane derivative,[8] containing two hydroxyl groups.

The ^1H NMR spectrum of **6** showed hydrogen signals at δ_H 0.70 (s), δ_H 0.93 (s), δ_H 0.97 (s), δ_H 1.01 (s), δ_H 1.17 (s), δ_H 1.18 (s) and δ_H 0.87 (d; *J* 7.0 Hz) that were associated to seven methyl groups. According to the literature,[8] the doublet at δ_H 0.87 is consistent with methyl group H-23 of members of the friedelane series.

In the ^1H NMR spectrum, the hydrogen signals at δ_H 3.12 (d; *J* 11.2 Hz) and δ_H 3.64 (d; *J* 11.2 Hz) were observed. Correlations of these signals with the signal of C-29 (δ_C 71.6) (CH_2) were observed in the HSQC contour map. The chemical shifts in this NMR region are typical of carbons attached to hydroxyl group,[14,16,19] suggesting the existence of hydroxyl group attached to C-29. Correlations observed in the HMBC contour map, among the signal of H-29 with signals of C-19 and C-30, confirmed the presence

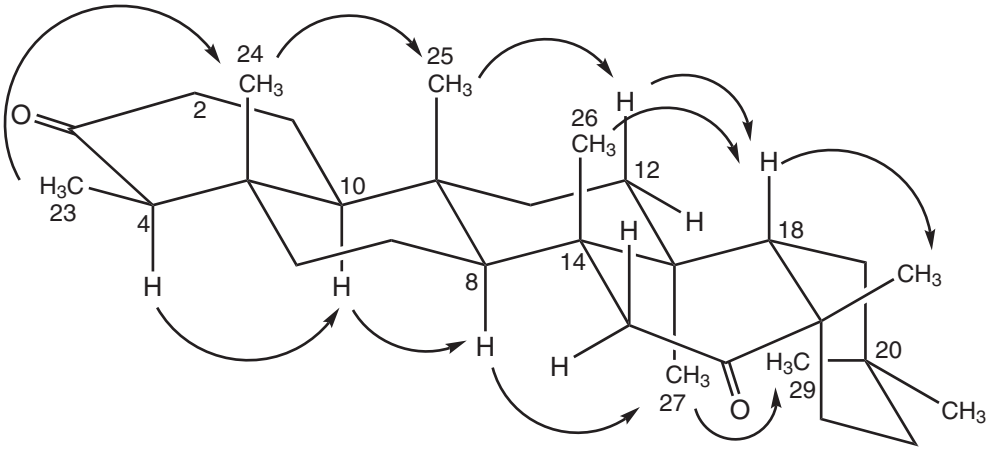


Figure 2. NOESY correlations observed for 3,16-dioxofriedelane (**5**).

Table 1. ^{1}H and ^{13}C NMR spectral data of 3,16-dioxofriedelane (**5**) and 3-oxo-12α,29-dihydroxyfriedelane (**6**)

	Triterpene **5**			Triterpene **6**		
entry	DEPT	δ_C	δ_H	DEPT	δ_C	δ_H
1	CH_2	22.2	1.69 ax; m 1.96 eq; m	CH_2	22.3	1.67 ax; m 1.93 eq; m
2	CH_2	41.4	2.30 eq; m 2.39 ax; m	CH_2	41.4	2.26 ax; dd; *J* 3.2 and 14.6 2.35 eq; dd; *J* 6.2 and 14.6
3	C	212.7	-	C	212.7	-
4	CH	58.1	2.24; m	CH	58.1	2.22; d; *J* 7.0
5	C	42.1	-	C	41.8	-
6	CH_2	40.9	1.25 ax; m 1.77 eq; m	CH_2	41.1	1.26 ax; m 1.75 eq; m
7	CH_2	18.6	1.38 eq; m 1.46 ax; m	CH_2	18.3	1.35 eq; m 1.53 ax; m
8	CH	52.3	1.46; m	CH	51.3	1.37; m
9	C	37.6	-	C	38.3	-
10	CH	59.2	1.55; m	CH	59.3	1.57; m
11	CH_2	35.3	1.56; m	CH_2	47.0	1.34 ax; m 1.75 eq; m
12	CH	29.0	1.43; m	CH	72.1	3.94; dd; *J* 4.4 and 11.2
13	C	39.1	-	C	44.8	-
14	C	40.4	-	C	41.1	-
15	CH_2	50.1	2.06 eq; m 2.39 ax; m	CH_2	31.6	1.44; m
16	C	219.0	-	CH_2	35.9	1.54; m
17	C	45.3	-	C	31.3	-
18	CH	43.9	2.08; m	CH	44.3	1.94 m
19	CH_2	35.4	1.23 eq; m 1.34 ax; m	CH_2	31.7	2.26 eq; m 1.75 ax; m
20	C	27.6	-	C	33.3	-
21	CH_2	31.6	1.48; m	CH_2	29.9	1.40 ax; m 1.26 eq; m
22	CH_2	30.7	1.72; m	CH_2	38.1	0.96 eq; m 1.75 ax; m
23	CH_3	6.8	0.88; d; *J* 6.8	CH_3	6.8	0.87; d; *J* 7.0
24	CH_3	14.6	0.74; s	CH_3	14.6	0.70; s
25	CH_3	17.3	0.90; s	CH_3	19.2	0.93; s
26	CH_3	20.3	1.20; s	CH_3	18.7	0.97; s
27	CH_3	16.2	0.89; s	CH_3	11.6	1.17; s
28	CH_3	27.3	1.30; s	CH_3	31.7	1.18; s
29	CH_3	31.0	1.05; s	CH_2	71.6	3.12 a; d; *J* 11.2 3.64 b; d; *J* 11.2
30	CH_3	35.2	0.96; s	CH_2	29.2	1.01; s

(δ values in ppm, *J* Hz, $CDCl_3$).

of a hydroxyl attached to C-29. By comparison of spectral data of **6** with similar skeleton reported in the literature,[8] it was also possible to locate the signal of C-12 (δ_C 72.1) and observe, in HMBC map, correlations of H-12 with C-27 (δ_C 11.6).

The detailed analysis of 2D NMR spectra and comparison with literature[8] allowed the assignment of all ^{1}H and ^{13}C chemical data of triterpene **6.** The following sequence of long range correlations (HMBC), H-23 (δ_H 0.87) ↔ C-5 (δ_C 41.8) ↔ H-24 (δ_H 0.70) ↔ C-10 (δ_C 59.3) ↔ H-25 (δ_H 0.93) ↔ C-8 (δ_C 51.3) ↔ H-26 (δ_H 0.97) ↔ C-13 (δ_C 44.8) ↔ H-27 (δ_H 1.17) ↔ C-18 (δ_C 44.3) ↔ H-28 (δ_H 1.18) was consistent with a friedelane type triterpene skeleton.[12,14,16,19]

The stereochemistry of triterpene **6** was established by means of data obtained from NOESY spectrum. It was possible to observe nOe correlations between H-23 and H-4ax, H-2eq, H-6eq and H-24. nOe effects were also observed between H-24 and H-1ax, H-6eq and H-25, between H-25 and H-11eq and H-12ax. By these NOESY correlations the establishment of a chair conformation for rings B and C (Figure 3) was enabled.

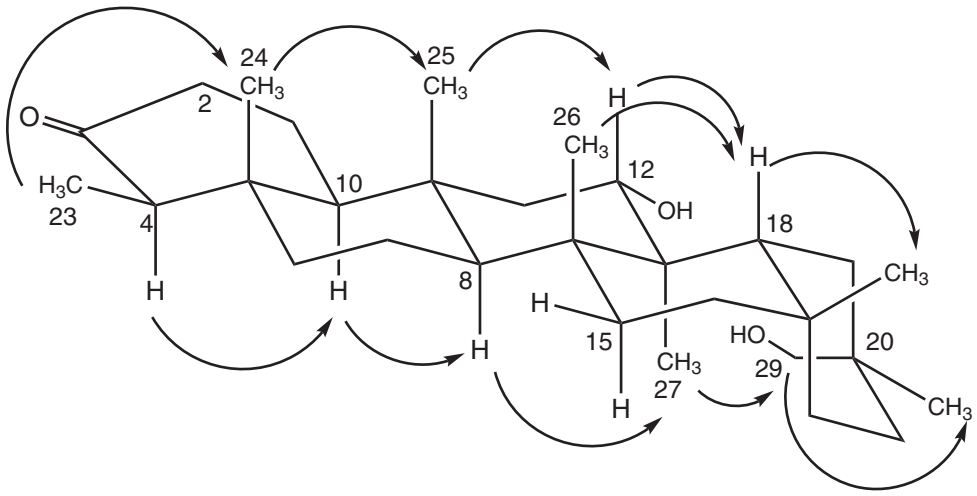


Figure 3. NOESY correlations observed for 3-oxo-12α,29-dihydroxyfriedelane (**6**).

In the NOESY contour map, it was observed that the signal of H-18 (δ_H 1.94) is correlated with the signals of H-12ax (δ_H 3.94) and H-26 (δ_H 0.97). The H-12 (δ_H 3.94) showed correlations between the signals of H-25 (δ_H 0.93) and H-26 (δ_H 0.97), indicating that H-12 is in axial position, and consequently the ring D of this new friedelane structure is in a chair conformation. Other evidence that confirms this hypothesis is the correlation between H-8 (δ_H 1.37) with H-27 (δ_H 1.17), which is only possible for ring D in chair conformation.

Correlations between the signal of H-29a (δ_H 3.12) with the signal of H-30 (δ_H 1.01), H-21 (δ_H 1.26) and H-15ax (δ_H 2.26) were also observed. The signal at δ_H 3.64 (H-29b) is correlated with the signals of H-27 (δ_H 1.17) and H-22 (δ_H 1.75). The NOESY data allowed determining that ring E also has chair conformation (Figure 3).

Antimicrobial activities have been described for pentacyclic triterpenes, such as oleananes,[20] ursanes,[20] friedelanes,[21] and lupanes.[22] It is speculated that the mechanism of action of triterpenes is due to a disruption on the microorganism's cellular membrane.[20,23] For this reason, the hexane extract and triterpenes 3-oxofriedelane (**1**), 3-oxo-12α-hydroxyfriedelane (**3**), 3,16-dioxofriedelane (**5**) and 3-oxo-12α,29-dihydroxyfriedelane (**6**) were tested against standard bacteria strains of *Escherichia coli, Citrobacter freundii, Bacillus cereus* and against the yeast *Candida albicans*, using disk diffusion test. The hexane extract was moderately active on disk diffusion test as well as in the broth dilution test, presenting a MIC value of 512 μg/mL. From the tested triterpenes, 3-oxo-12α-hydroxyfriedelane (**3**) was active in the macrodilution test, presenting a MIC = 512 μg/mL against *C. albicans*. The activity of **3** was lower in comparison to that was produced by miconazole (16 μg/mL), and was not detected in the disk diffusion test, probably because of low polarity of this compound.[24]

Supplementary Information

Spectra (IR, 1D/2D NMR and EM) and Tables of antimicrobial test results are available free of charge at http://jbcs.org.br as PDF file.

Acknowledgments

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