Antimycobacterial Activity of Milemarinol, a New Squalene-Type Triterpene, and Other Isolate?

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Abstract

A new triterpene, named milemarinol (1), was isolated from Homalolepis suffruticosa Engl., Simaroubaceae, along with 10 known metabolites, chaparrinone (2), scopoletin (3), 5-methoxycanthin-6-one (4), eurylene (5), hispidol A (6), hispidol B (7), nilocitine (8), α-dihydronylocytine (9), β-dihydronylocytine (10), and teurilene (11). These compounds were characterized based on their spectral data, mainly 1D (1H, 13C-APT) and 2D (1H-1H-COSY, NOESY, HSQC, HMBC) NMR and their mass spectra (HR-ESI-MS), in comparison with data from the literature. Compounds 1 to 6, 8, and 9 were evaluated for their antimycobacterial activity against 2 strains (H37Rv and M299).

Keywords

Simaroubaceae, triterpenes, quassinoid, alkaloids, Mycobacterium

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The family Simaroubaceae contains 117 reported species of trees, whose characteristic is the bitter taste of their cortex.¹² This can be attributed to the presence of quassinoids, which is also considered a taxonomic marker of this family.³ The genus Homalolepis Turcz., recently segregated from Simaba Aub., comprises 28 species, spread mainly in tropical South America.²

Besides the quassinoids, other types of triterpenes, alkaloids, and other classes of compounds were also observed.⁴ These compounds showed many biological activities like antibacterial,⁵⁶ anticancer,⁷⁸ antileishmanial ⁹¹¹; antiviral,¹²¹⁴ and others.¹¹,¹⁵⁻¹⁹

Thus, in view of the high biological potential of the compounds of the Simaroubaceae, a phytochemical study of the roots of Homalolepis suffruticosa was carried out, during which a new unusual squalene triterpene, named milemarinol (1), was characterized and 10 known compounds were identified; their antimycobacterial and cytotoxicity activities were evaluated.

Results and Discussion

Elaboration of the MeOH and n-hexane extracts of the roots of H. suffruticosa through classical chromatographic methods resulted in the isolation of 11 compounds, ¹ to ¹¹, whose structures are shown in Figure 1. A new triterpenoid, named milemaronol,⁵ along with 10 known compounds, chaparrinone (²), scopoletin (³), ⁵⁻¹⁰ 5-methoxycanthin-6-one (⁴),²² eurylene (⁵),²³ hispidol A (⁶),²⁴ hispidol B (⁷),²⁴ nilocitine (⁸),²⁵ α-dihydronylocytine (⁹),²⁶ β-dihydronylocytine (¹⁰),²⁶ and meso-teurilene (¹¹),²⁷ was characterized based on their ¹H- and ¹³C-NMR spectral data, especially 2D-NMR, and by comparison of the mass spectral data with the literature values.

Milemarinol (1) was isolated as an yellow oil. Its HR-ESI-MS showed peaks due to the presence of a sodium ion at
m/z 515.3606 ([M+Na]+, calc. m/z 515.3712) and a potassium ion at m/z 531.3481 ([M+K]+, calc. m/z 531.3452) (Supplemental Scheme 1S). These data, combined with the information obtained by 1D and 2D NMR spectral analysis, were used to deduce the molecular formula C₃₀H₅₂O₅, indicating 5 degrees of unsaturation (2 C=C bonds and 3 rings).

The ¹³C-APT-NMR spectrum of 1 revealed signals corresponding to 30 carbon atoms, including 6 quaternary [C₆: 2 sp² (double bond) and 4 sp³ linked to oxygen atoms], 6 methines [(CH)₆: 4 sp³ carbinolics and 2 sp² olefinics at δC 124.8 and 124.5], 10 methylenes [(CH₂)₁₀], and 8 methyls [(CH₃)₈ including 4 linked to sp² carbon atoms (2 at δC 25.7 and 2 at δC 17.6)].

¹H NMR spectral analysis of 1 showed 2 triplet signals in the region of the olefinic hydrogens at δH 5.11 (H-3, t, 6.7) and 5.14 (H-22, t, 7.1); 4 double doublet signals in the region of carbinolic hydrogens δH 3.90 (H-7, dd, 7.8, 5.0), 3.69 (H-18, dd, 11.5, 3.9), 3.71 (H-11, dd, 12.1, 1.9), and 3.55 (H-14, dd, 7.6, 5.0); and 4 singlet signals corresponding to methyl groups linked to sp³ carbon atoms at δH 1.71 (3H-24), δH 1.69 (3H-1), δH 1.64 (3H-25), and δH 1.62 (3H-30). All ¹H and ¹³C chemical shifts are assigned in Table 1.

The location of these 2 C=CH bonds was established through analysis of the HMBC spectrum, which revealed cross-peaks of δC 131.3 (C-2) with 3H-1 (δH 1.69, 2JCH), 3H-25 (δH 1.64, 2JCH), and 2H-4 (δH 2.08, 3JCH) and δC 131.9 (C-23) with 3H-24 (δH 1.71, 2JCH), 3H-30 (δH 1.71, 2JCH), and 2H-21 (δH 2.03, 1.78, 3JCH). Additional HMBC couplings via 3JCH of CH-3 (δC 124.8) with 2H-5 (δH 2.08) and CH-22 (δC 124.5) with 2H-20 (δH 1.90, 1.75) confirmed the terminal units (Me₂C=CH-CH₂-CH₂-5 and Me₂C=CH-CH₂-CH₂-20). The tetrahydrofuran ring was observed based on the carbon shift of δC 85.2) with cross-peak with H-7 (δH 3.90, 2H-9 (δH 2.25 and 1.48), H-11 (δH 3.71), and H-27 (δH 1.14). This allowed to recognize the identical unit from C-1 to C-10 of 11, confirmed by comparative analysis of spectral data.

In the region of the carbinolic carbons of 1, signals were observed at δC 73.8 (C-6) and δC 73.2 (C-19), which displayed correlations in the HMBC spectrum with methyl groups 3H-26 (δH 1.71, 2JCH) and 3H-30 (δH 1.71, 2JCH), and 2 carbinolic hydrogens H-7 (δH 3.90, dd, 7.8, 5.0) and H-18 (3.69, dd, 11.5, 3.9), which showed a system with a methyl linked to a carbinolic carbon previously observed in the equivalent spectra of eurylene and teurilene. Moreover, in the region of the carbinolic carbons, signals were observed at δC 84.4 (CH-7), 85.2 (C-10), 75.1 (CH-11), 78.0 (CH-14), 75.9 (C-15), and 72.9 (C-18), corresponding to an ether function.

The location of this unit linked to C-11 (δC 75.1) was deduced by heteronuclear interaction of this nonprotonated carbon with...
3H-27 (δH 1.90, JCH = 7.1 Hz). The other terminal unit involving carbon atom C-19 was linked to CH-18 (δC 72.9, CH2-12 (δH 1.20)) with 3H-27 (δH 3.55). The HMBC correlation (JCH) of this methinic carbon with 3H-29 (δH 1.24) with H-7 (δH 3.90); 3H-27 (δH 1.14) with H-7 (δH 3.90), H-11 (δH 3.71), and H-14 (δH 3.55); and 3H-28 (δH 1.20) with H-14 (δH 3.55).

The antymycobacterial activity against Mycobacterium tuberculosis strains H37Rv and M299, and cytotoxic activities of compounds 1 to 6, 8, and 9 were evaluated; the results are shown in Table 2. Compounds 2 and 5 showed excellent growth inhibition of 2 Mycobacterial strains, but revealed toxicity in viable cells. Compounds 4, 6, and 9 yielded a good response in the inhibition of both strains, but high cytotoxicity. On the other hand, compound 1 showed the best result, being sensitive to both mycobacteria and presenting low cell toxicity (Table 2).

### Material and Methods

#### General Experimental Procedures

Column chromatography (CC) was performed on silica gel 60 (0.063-0.200 mm, Merck), and n-hexane (98.5%), methanol (99.8%), ethyl acetate (99.5%), and dichloromethane (99.5%) were used as mobile phase solvents, purchased from Synth (São Paulo, Brazil). 1D and 2D NMR analysis was performed on a 500 MHz Bruker Ascend 500 NMR spectrometer operating at 500 MHz for 1H NMR and 125 MHz for 13C NMR. Deuterated chloroform (CDCl3), tetrahydrofuran (THF), dichloromethane, dimethyl sulfoxide (DMSO), methanol-d4, and pyridine-d5, 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.

#### Plant Material

Roots of *H. suffruticosa* were collected in September 2017, in Araguari City, Minas Gerais. The species was identified by the taxonomist José Rubens Pirani from the Universidade de São Paulo (USP). A voucher specimen (HUENF-10840) was deposited in the herbarium of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF).

#### Extraction and Isolation

*Homalodisca suffruticosa* roots were dried and powdered. The extraction was performed first with n-hexane (CH, 20.5 g) and
then with methanol (CM, 23.4 g). The methanolic extract (23.4 g) was fractionated by silica gel CC, with a polar gradient of CH₂Cl₂:MeOH, obtaining 9 fractions (CM1-CM9). CM4 was similarly rechromatographed, generating 10 fractions (CM4.1-CM4.10). Chaparrinone (2) and scopoletin (3) were identified in fractions CM4.7 and CM4.4, respectively. Fraction CM4.8 was also chromatographed with hexane:acetone, generating 17 fractions (CM4.8.1-CM4.8.17); fractions CM4.8.3 (30.3 mg) and CM4.8.11 were identified as milemaronol (1) and 5-methoxicantin-6-one (4), respectively.

The hexane extract (CH, 20.5 g) was fractionated by silica gel CC with a gradient of hexane:acetone to yield 14 fractions (CH1-CH14). The CH5 fraction was similarly rechromatographed, yielding 9 fractions (CH5.1-CH5.9). Nilocitine (8) was identified in fraction CH5.3 (4.5 mg). The CH8 fraction (1.9 g) was subjected to CC with CH₂Cl₂:MeOH as eluent, yielding 10 fractions (CH8.1-CH8.10). In the CH8.5 fraction, a precipitate was observed, which was recrystallized and identified as eurylene (5). Fraction CH8.5 was chromatographed with hexane:EtOAc yielding α-dihydronylocytin (9) and β-dihydronylocytin (10) as a mixture. CH10 (1.46 g) was chromatographed with hexane:EtOAc, obtaining 8 fractions (CH 10.1-CH10.8). Hispidol A (6) and hispidol B (7) were identified as a mixture in CH10.3 (24.9 mg), and compound 6 was isolated from CH10.4 (14.5 mg). Meso-teurilene (11) was isolated from CH12 (29.1 mg).

**Culture of Mycobacteria and Evaluation of Bacterial Growth**

Two strains of *Mycobacterium tuberculosis* were used in this study (a virulent laboratory strain H37Rv, ATCC 27294 and a highly virulent Mtb strain Beijing M299, isolated from a TB patient in Mozambique), which were evaluated for virulence in a previous study. Middlebrook 7H9 broth, containing 10% dextrose albumin complex (ADC), 0.5% glycerol, and 0.05% Tween-80, was used for the growth of the mycobacterial strains at 37°C, under conditions of containment of Biosafety 3. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed to quantify bacterial growth, using the procedures described by Ventura et al. The samples were analyzed by optical density at 570 nm. For negative control, untreated bacterial suspensions were used, while for positive control rifampicin was applied.

**Evaluation of Cytotoxicity by MTT Assay**

RAW 264.7 macrophages were treated with the compounds at concentrations of 4, 20, 100, and 500 µg/mL. After 24 hours, the levels of cytotoxicity of the samples were assessed using mitochondrial functionality using the MTT method and compared with negative (macrophages stimulated by lipopolysaccharide - LPS) and positive (macrophages stimulated by LPS and treated with 1% Triton X-100) controls. Values are reported as mean ± standard deviation, and different groups were considered significant according to $P < .001$ (***) and $P < .05$ (*).

| MIC (µg/mL) | IC (µg/mL) |
|------------|------------|
| H37Rv      | M299       | MTT        |
| 1          | 42.1 ± 0.7 | 64.4 ± 0.8 | 307.7 ± 0.1 |
| 2          | 3.0 ± 1.2  | 7.6 ± 1.4  | 3.95 ± 1.0  |
| 3          | ≥500       | ≥500       | ≥500        |
| 4          | 30.4 ± 0.5 | 34.9 ± 0.3 | 46.9 ± 0.1  |
| 5          | 1.4 ± 0.9  | 2.0 ± 0.6  | 40.1 ± 0.7  |
| 6          | 14.6 ± 1.9 | 7.2 ± 1.9  | 3.4 ± 0.3   |
| 8          | ≥500       | 253.8 ± 1.0 | 79.5 ± 0.1  |
| 9          | 25.5 ± 0.7 | 24.4 ± 0.7 | 51.6 ± 0.2  |
| Rifampicin | 0.2 ± 0.1  | 1.1 ± 0.1  | -           |

**Figure 2.** Important ¹H-¹H-NOESY correlations for milemaronol (1).
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