New hydroperoxycycloartane—complete chemical shifts assignment of $^{13}$C and $^1$H—and cytotoxicity evaluation of cycloartanes isolated from *Trichilia casaretti*

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**ABSTRACT**

New cycloartane, 22-hydroxy-25-hydroperoxycycloart-23E-en-3-one (1), along with six known analogues (2–7) and three steroids (8–10), were isolated from the leaves of *Trichilia casaretti*. Structures were elucidated mainly on the basis of the analysis of 1D and 2D NMR ($^1$H and $^{13}$C) and HREIMS spectroscopic data, involving comparison with data of the literature. The cytotoxic activities of 1–7 and 10 isolated compounds were also evaluated against human leukemia cell line Molt-4 (acute lymphoblastic) and exhibited good cytotoxic activity with IC$_{50}$ values ranging from 10.62 to 21.14 $\mu$M.
1. Introduction

*Trichilia* genus belongs to the Meliaceae family (also known as Mahogany), consists of 98 species distributed in tropical and subtropical areas (Patrício and Cervi 2005). Researches constantly shown the presence of secondary metabolites of the metabolic pathway of terpenoids, especially limonoids (Ji et al. 2015a; Liu et al. 2016; Wang et al. 2017). These compounds are considered as chemosystematic markers of the family (Vieira et al. 2014). In addition, these species present several biological activities, as in the case of *T. americana* showed the cytotoxic activities of isolated compounds against five human tumour cell lines (Ji et al. 2015b). In Brazil it has several species of the *Trichilia* genus. Particularly, *Trichilia casaretti* (distributed in the Atlantic Forest) has already identified cycloartanes and sesquiterpenes in their chemical composition with biological activity (Vieira et al. 2010; Vieira et al. 2018). In this study, we investigated the constituents from *T. casaretti* leaf where led to identification of one new cycloartane (1), also six know analogues (2–7) and three steroids (8–10), showed in Figure 1, as well as evaluating cytotoxic activity against leukemia cell line Molt-04.

2. Results and discussion

The analysis of $^1$H NMR spectra of compounds 1–7 showed signals suggesting the presence of cycloartane skeleton by two doublets characteristic of 2H-19 (cyclopropane ring) at $\delta_H 0.39 \sim 0.80$ ($J = 4.2$ Hz). The cycloartanes with keto group in C-3 (1–3) presented of two doublets ($d, J = 4.2$ Hz) at $\delta_H 0.80$ and 0.59 and with CH-3 sustaining hydroxyl group (4–7) the doublets ($d, J = 4.4$ Hz) at $\delta_H 0.58$ and 0.36.

Compound 1 was obtained as a amorphous powder. The molecular formula was determined as C$_{30}$H$_{48}$O$_4$ by HRESIMS m/z 495.3391 ([M + Na]$^+$, calcd for C$_{30}$H$_{48}$O$_4$Na$^+$.

![Figure 1. Structures of compounds 1-10.](image-url)
m/z 495.3450), indicating fourteen hydrogen deficiency (C$_{30}$H$_{62}$O$_4$–C$_{30}$H$_{48}$O$_4$ = 14) corresponding to seven degree of unsaturation. The $^{13}$C-APT NMR spectrum of 1 revealed $^{13}$C signals corresponding to 30 carbon atoms: seven non-hydrogenated [including one sp$^2$ carbonylic at $\delta_{C}$ 216.7 (O = C-3) and one sp$^3$ oxygenated at $\delta_{C}$ 81.9 (O-C-25)], seven methines [including one sp$^3$ oxygenated at $\delta_{C}$ 74.5 (O-CH-22) and two sp$^2$ olefinics at $\delta_{C}$ 129.3 (=CH-23) and 136. (=CH-24) = HC = CH], nine methylenes [including one cyclopropane at $\delta_{C}$ 29.5 (CH$_2$-19) above referenced] and seven methyl groups, allowing to deduce the expanded partial molecular formula C$_{30}$H$_{46}$O$_3$ requesting two hydrogen atoms and one oxygen atom to confirm the molecular formula C$_{30}$H$_{48}$O$_4$ established by HRESIMS (Scheme S1). Thus, the seven degree of unsaturation were attributed to one carbonyl group (C-3), one double bond (23-CH = HC-24), one cyclopropane ring and more four rings, compatible with cycloartane skeleton.

The HSQC spectrum of 1 was possible to confirm $^{13}$C signals from seven non-hydrogenated carbons by the predicted absence of cross-peaks [e. g. carbonyl $\delta_{C}$ 216.7 (C-3) and an oxygenated quaternary carbon $\delta_{C}$ 81.9 (C-25)] corresponding to heteronuclear correlations with signals of hydrogen atoms. The cross-peaks involving direct correlations via one bond ($^1$J$_{CH}$) of $^{13}$C and $^1$H signals were observed to methines [e. g.: double bond: $\delta_{C}/\delta_{H}$ 136.0/5.82 (d, J = 15.9 Hz, trans coupling, CH-24) and 129.3/5.73 (dd, J = 15.9 and 6.9 Hz, CH-23)], and oxygenated carbon at $\delta_{C}/\delta_{H}$ 74.5/4.26 (dd, J = 6.9 and 3.9 Hz, CH-22)], methylenes and methyls (Table S1). Farther cross-peaks observed in the HSQC of 1 were summarized in Table S1.

Analysis of the $^1$H NMR data displayed signals to six terciary [$\delta_{H}$ 0.87 (3H-18), 1.38 (3H-26), 1.37 (3H-27), 1.06 (3H-28), 1.11 (3H-29) and 1.05 (3H-30)] and one secondary [$\delta_{H}$ 0.93 (d, 6.6, 3H-21)] methyl groups (Table S1). The cyclopropane ring was deduced from the two doublets at $\delta_{H}$ 0.80 (d, 4.2, H-19x) and 0.59 (d, 4.2, H-19z). It was also possible to observe two signals referring to double-bond hydrogens at $\delta_{H}$ 5.82 (d, 15.9, H-24) and 5.73 (dd, 15.9, 6.9, H-23).

The HMBC spectrum of 1 showed correlations of the signals from the H-2, H-5, 3H-28 and 3H-29 with the carbon signal at $\delta_{C}$ 216.7 suggesting the presence of carbonyl in C-3. While further HMBC correlations from the 3H-21, H-23 and H-24 to $\delta_{C}$ 74.5 indicated the hydroxyl was located at C-22. Beyond the correlations between H-23, H-24, 3H-26 and 3H-27 to $\delta_{C}$ 81.9 hinted the presence of oxygenated quaternary carbon in C-25. Analysis of the $^1$H-$^1$H COSY spectrum gave partial structures, 2H-1/2H-2, H-5/2H-6/2H-7/H-8, 2H-11/2H-12, and H-15/2H-16/H-17/H-20/H-21 and H-20/H-22/H-23/H-24. Figure S1 showed the principal HMBC and $^1$H-$^1$H COSY correlations. Additional heteronuclear long-range couplings ($^2$J$_{CH}$ and $^3$J$_{CH}$) revealed by HMBC are summarized in Table S1.

The hydroperoxy group (HOOC-25) of 1 was deduced by comparison of the $^{13}$C NMR spectra of 1 and 2 (HOC-25). The protection $\gamma$ effects observed in the $^{13}$C chemical shifts signals of CH-24 ($\Delta \delta_{C}$ = 4.4 ppm), CH$_3$-26 ($\Delta \delta_{C}$ = 5.5 ppm) and CH$_3$-27 ($\Delta \delta_{C}$ = 5.6 ppm) and deprotection $\beta$ effect in CH-23 ($\Delta \delta_{C}$ + 4.2 ppm) and C-25 ($\Delta \delta_{C}$ + 11.1 ppm) were used to recognize the presence of the hydroperoxy group no carbon atom C-25 of 1 (Figure S2). In addition, the molecular formula was also used to confirm the presence of the hydroperoxy group (Scheme S1).
The $^1$H-$^1$H NOESY spectrum of 1 revealed strong NOE dipolar interactions of 3H-29 ($\delta_H$ 1.11) with H-19β ($\delta_H$ 0.59), 3H-29 with H-8 ($\delta_H$ 1.60), H-8 with H-19α ($\delta_H$ 0.80) and 3H-18 ($\delta_H$ 0.87), H-5 ($\delta_H$ 1.73) with 3H-30 ($\delta_H$ 1.05) and H-17 ($\delta_H$ 1.54) with 3H-21 ($\delta_H$ 0.93) which allowed to deduce important stereochemistry summarized in Figure S3.

In addition to new cycloartane, 22-hydroxy-25-hydroperoxycycloart-23-en-3-one (1), six other known analogues and three steroids were isolated and identified as 22,25-dihydroxycycloart-23-en-3-one (2) (Lago and Roque 2002), 22-hydroxy-cycloartenone (3) (Bohlmann et al. 1985), 22-hydroxycycloartenol (4) (Bohlmann et al. 1985), 24-methylencycloartan-12-oxo-3,22-diol (5) (Vieira et al. 2018), cyclokirilidiol (6) (Kimura et al. 1997) and isocyclokirilidiol (7) (Kimura et al. 1997), β-sitosterol (8) (Chaturvedula and Prakash 2012), stigmasterol (9) (Chaturvedula and Prakash 2012) and itesmol (10) (Zanno et al. 1973), by comparing their experimental NMR (1D and 2D) and literature data.

Subsequently were determined the inhibitory effects of 1–7 and 10 to human leukemia cell line Molt-4 (acute lymphoblastic) and the IC$_{50}$ values of these compounds were evaluated based on MTT assay results as summarized in Table S2. As results, all tested compounds exhibited good cytotoxic activity comparable to the cisplatin positive control. Besides, compounds 1 and 3 were the ones that showed the best result with IC$_{50}$ values 11.28 and 10.62 μM, respectively, followed to compounds 2 (15.84 μM), mixture of 6 and 7 (12.76 μM), and 10 (14.03 μM). Additionally, compounds 4 (20.42 μM) and 5 (21.14 μM) were the ones that presented results similar to those of cisplatin.

3. Experimental
3.1. General procedures
Infrared (IR) spectra were recorded with a Shimadzu FT-IR 8300 spectrophotometer. NMR spectra were acquired by using Bruker Ascend 500 (500 MHz for $^1$H and 125 MHz for $^{13}$C) in CDCl$_3$. Chemical shifts ($\delta$) in ppm and coupling constants (J) in Hz. HRESI data were acquired on a micrOTOF-Q II Bruker Daltonics, with the use of the positive ion mode of analysis. Column chromatography (CC) was performed on silica 60 (0.063–0.200 mm, MERCK) and for the preparative thin layer chromatography (PTLC) was used silica gel 60 PF$_{254}$ (MERCK) on glass plates. TLC plates contained with silica gel 60 F$_{254}$ (MERCK) were used for analytical analysis; detection was under UV (254 and 365 nm) and chromogenic reagent (Vanillin/sulfuric acid). The solvents used were n-Hexane, methanol (MetOH), ethyl acetate (AcOEt), dichloromethane (CH$_2$Cl$_2$) and n-butanol purchased from Synth (São Paulo, Brazil).

3.2. Plant material
The leaf of Trichilia casaretti C. DC., Meliaceae, were collected in Reserva Natural Vale, Linhares City, Espirito Santo state, Brazil. A voucher specimen (CVRD-449) was deposited in the herbarium of the Reserva Natural Vale.
3.3. Extraction and isolation

Powdered leaf of *T. casaretti* (1.5 kg) were soaked in MeOH (three times, in the interval of 2, 5 and 7 days). Then, the extract was suspended in H$_2$O:MeOH (3:1) and partitioned using CH$_2$Cl$_2$, AcOEt, ButOH, respectively. The fractions Fr1-Fr15 was obtained from CH$_2$Cl$_2$ partition (17,857 g) by silica gel column chromatography (CC) employing Hexano:AcOEt as the elution solvent. Fraction Fr6 (844.3 mg) was recrystallized from acetone to afford mixture of compounds 8 and 9 (256 mg). Fr8 (482.3 mg) fraction was chromatographed on a silica gel column and eluted with a gradient of Hexano:AcOEt yielding 12 fractions. Compound 5 (7 mg) was identified from fraction Fr8. The Fr8.4 (50 mg) fraction was rechromatographed similarly, obtaining compound 3 (5 mg). Fraction Fr8.11 (126 mg) was chromatographed on a silica gel column and eluted with a gradient of Hexano:AcOEt to obtain compounds 1 (8 mg) and 2 (15 mg). Fraction Fr10 (366 mg) was rechromatographed similarly yielding compound 4 (6 mg). Compound 10 (16 mg) was obtained from fractionation of Fr11 (2.1892 g) using CC on silica gel column and eluted with gradient hexane:AcOEt. Fr13 (572.3 mg) was subjected to CC on silica gel eluting with gradient hexane:AcOEt to give 6 fractions. The mixture of compounds 6 and 7 (5 mg) was further purified from Fr13.6 (125 mg) using CC on silica gel and hexane: AcOEt as eluents.

22-hydroxy-25-hydroperoxycycloart-23-en-3-one (1): Amorphous powder; IR (KBr, disc) $\nu_{\text{max}}$ (cm$^{-1}$): 3435, 2966, 2938, 1705, 1381, 734; $^1$H NMR (500 MHz): $\delta$H = 5.82 (d, $J = 15.9$ Hz, 1H, H-24), 5.73 (dd, $J = 15.9$, 6.9 Hz, 1H, H-23), 4.26 (dd, $J = 6.9$, 3.6 Hz, 1H, H-22), 2.72 (td, $J = 13.7$, 6.3 Hz, 1H, Ha-2), 2.32 (dt, $J = 13.7$, 3.3 Hz, 1H, Hb-2), 2.05 (m, 3H, Ha-11 and 2H-16), 1.87 (m, 1H, Ha-7), 1.86 (m, 1H, Ha-1), 1.82 (m, 1H, H-20), 1.73 (dd, $J = 12.2$, 4.2 Hz, 1H, H-5), 1.62 (m, 2H, 2H-15), 1.60 (m, 1H, H-8), 1.56 (m, 1H, Hb-1), 1.55 (m, 2H, 2H-6), 1.54 (m, 1H, H-17), 1.38 (s, 3H, 3H-26), 1.37 (s, 3H, 3H-27), 1.37 (m, 1H, H-12), 1.22 (m, 1H, Hb-7), 1.18 (m, 1H, Hb-11), 1.11 (s, 3H, 3H-29), 1.05 (s, 3H, 3H-30), 0.93 (d, $J = 6.6$, 3H, 3H-21), 0.87 (s, 3H, 3H-18), 0.80 (d, $J = 4.2$, 1H, Ha-19), 0.59 (d, $J = 4.2$, 1H, Hb-19) ppm; $^{13}$C NMR (500 MHz): $\delta$C = 216.7 (C-3), 136.0 (C-24), 129.3 (C-23), 81.9 (C-25), 74.5 (C-22), 50.2 (C-4), 49.2 (C-17), 48.4 (C-5), 47.8 (C-8), 45.6 (C-13), 45.6 (C-14), 42.2 (C-20), 37.4 (C-2), 35.6 (C-12), 33.4 (C-1), 32.7 (C-15), 29.5 (C-19), 27.4 (C-7), 26.6 (C-16), 26.0 (C-10), 25.9 (C-11), 24.6 (C-26), 24.3 (C-27), 22.2 (C-28), 21.5 (C-6), 21.1 (C-9), 20.7 (C-30), 19.2 (C-29), 18.1 (C-18), 12.1 (C-21); HRESIMS m/z 495.3391 [M + Na]$^+$ (calcd m/z for C$_{30}$H$_{48}$O$_4$Na$^+$–m/z 495.3450

3.4. Antineoplastic activity

The experimental procedures to antineoplastic activity were presented in the supporting information.

4. Conclusions

An investigation of the leaves of *Trichilia casaretti* resulted into isolation of a new cycloartane, 22-hydroxy-25-hydroperoxycycloart-23-en-3-one (1) together with nine known compounds (2–10). The structure of 1 was characterized using NMR and Mass spectroscopic data. The compounds have shown good cytotoxic activity against
human leukemia cell line Molt-4 (acute lymphoblastic). Besides, the known compounds 2, 3, 6 and 7 are reported for the first time from the genus *Trichilia*.

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