

New cytotoxic limonoids from the stem bark of *Chisocheton pentandrus* (Blanco) Merr

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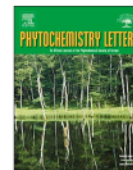
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New cytotoxic limonoids from the stem bark of *Chisocheton pentandrus* (Blanco) Merr

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ABSTRACT

Three new limonoids namely pentandricines B–D (1–3), were isolated from *n*-hexane extract of the stem bark of *Chisocheton pentandrus*, along with four known limonoids (4–7). The chemical structures of pentandricines B–D, were fully elucidated based on UV, IR, HR-TOFMS, 1D- and 2D-NMR spectra. Furthermore, compounds 1–7 were evaluated for their cytotoxic activity against MCF-7 breast cancer cells *in vitro*, with compound 7 (nimonol) showing strongest cytotoxicity activity with an IC₅₀ value of 22.03 ± 0.026 μM.

1. Introduction

Limonoids from the genus *Chisocheton* have been subjected to various studies, due to their interesting biological activities such as insecticidal, insect antifeedant, antibacterial, antifungal, antimalarial, anticancer and antiviral (Chong et al., 2019; Shilpia et al., 2016; Wong et al., 2011; Katja et al., 2017; Mohamad et al., 2009; Tan and Luo, 2011; Bordoloi et al., 1993). In addition, the structural diversity in limonoids makes it interesting and compelling for investigation (Chong et al., 2012; Najmuldeen et al., 2012; Awang et al., 2007; Chan et al., 2007; Roy and Saraf, 2006; Connolly et al., 1979). The genus *Chisocheton* produces tirrucalane-type triterpenoids (Yang et al., 2009), lanostane-type triterpenoids (Katja et al., 2017), dammarane-type triterpenoids (Chan et al., 2012) and euphane type-triterpenoid (Supratman et al., 2019). In the search for cytotoxic limonoids from Indonesia *Chisocheton* species, a mexicanolide-type limonoid, dysobinol; from the bark of *C. macrophyllus* (Nurlelasari et al., 2017; Katja et al., 2016), a trijugin-type limonoid known as chisotrijugin; from the bark of *C. cumingianus* (Katja et al., 2017) and a vilacinine-type limonoid known as pentandricine from the bark of *C. pentandrus* (Supriatno et al., 2018) have been reported. In this paper, we reported the isolation and structural elucidation of three new azadirone-type limonoids,

pentandricines B–D (1–3) and four known limonoids (4–7), along with their cytotoxic activity against MCF-7 breast cancer cells.

2. Results and discussion

The *n*-hexane extract from the bark of *C. pentandrus* was first fractionated over a vacuum-liquid chromatographed (VLC) column packed with silica gel 60 by gradient elution. The VLC fractions were then separated using reverse phase column chromatography, as well as preparative TLC on silica gel GF₂₅₄ to give seven compounds, 1–7 (Figs. 1 and 2 Fig. S1).

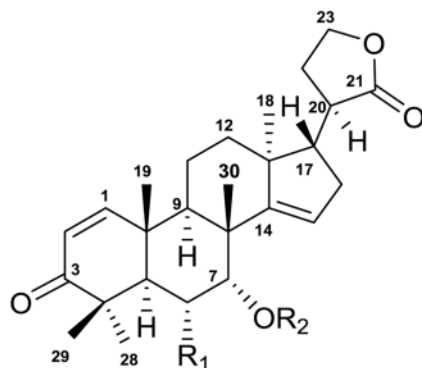
Pentandricine B (1) was isolated as a colorless amorphous solid, with its molecular formula established as C₂₈H₃₈O₅ from a combined analysis of the HR-TOFMS spectra *m/z* 454.2871 [M+H]⁺, and NMR data (Table 1), thus requiring ten index of hydrogen deficiency. The UV spectrum showed absorption maximum at 240 nm (log ε 4.2), indicating the presence of an enone group. The IR spectrum showed bands which were ascribed to an olefinic C–H stretching (ν_{max} 3117 cm^{−1}), aliphatic C–H stretching (ν_{max} 2944 cm^{−1}), carbonyl group of a lactone (ν_{max} 1775 cm^{−1}) and conjugated ester (ν_{max} 1722 cm^{−1}). The IR spectrum also showed an α,β-unsaturated carbonyl (ν_{max} 1665 cm^{−1}), isolated double bond (ν_{max} 1565 cm^{−1}) and ether groups (ν_{max} 1165 cm^{−1}).

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1. $R_1 = H$; $R_2 = Ac$
2. $R_1 = R_2 = H$
3. $R_1 = OAc$; $R_2 = Ac$

Fig. 1. Structures of Compounds 1–3.

Further, the 1H -NMR spectrum showed six tertiary methyls at δ_H 1.00 (3H, s, Me-18), 1.06 (3H, s, Me-28), 1.06 (3H, s, Me-29), 1.17 (3H, s, Me-19), 1.17 (3H, s, Me-30) and 1.94 (3H, s, Me-2'), of which the tertiary methyls were typical for azadirone-type limonoid (Lavie et al., 1971). Three α,β methine protons at δ_H 5.29 (1H, dd, $J = 1.8$, 3.6 Hz, H-15), 5.85 (1H, d, $J = 10.8$ Hz, H-2) and 7.13 (1H, d, $J = 10.8$ Hz, H-1), an oxygenated methine proton at δ_H 3.92 (1H, m, H-23a) and 4.46 (1H, m, H-23b), one oxygenated methine proton at δ_H 5.23 (1H, t, $J = 2.4$ Hz, H-7) and some aliphatic signals in the upfield region were also observed in the 1H -NMR spectrum. The ^{13}C NMR / DEPT spectra revealed twenty-eight carbons consisting of an α,β -unsaturated carbonyl at δ_C 204.6, carbonyl lactone at δ_C 176.1, carbonyl ester at δ_C 170.2, six methyls at δ_C 19.1 (Me-18), 20.0 (Me-19), 21.2 (Me-2'), 21.4 (Me-29), 27.1 (Me-28) and 27.5 (Me-30). The ^{13}C NMR spectrum also revealed three sp^2 methine carbons at δ_C 157.9 (C-1), 125.5 (C-2) and 119.7 (C-15), one sp^2 quaternary carbon at δ_C 158.8 (C-14), an oxygenated methylene carbon at δ_C 72.5 (C-23), an oxygenated methine carbon at δ_C 74.4 (C-7), and five sp^3 methylenes at δ_C 16.5 (C-11), 23.8 (C-6), 33.8 (C-12), 34.1 (C-22) and 34.9 (C-16), four sp^3 methines at δ_C 37.6 (C-20), 38.4 (C-9), 46.2 (C-5) and 58.2 (C-17). These functionalities accounted for five out of the ten index of hydrogen deficiency, while the remaining five correspond to the pentacyclic limonoid structure (Supriatno et al., 2018; Najmuldeen et al., 2012). Comparison of the NMR spectra of 1 with those of apoephrol-type limonoid isolated from *C. microcarpus* (Gunning et al., 1994), showed that compound 1 has identical A, B, C and D rings, with an acetate substituent at C-7. In compound 1, instead of the typical 4-substituted- γ -lactone moiety, the NMR spectra displayed signals for 3-substituted- γ -lactone group [δ_H 2.70 (1H, m, H-20), 2.17 (1H, m, H-22a), 2.20 (1H, m, H-22b), 3.92 (1H, m, H-23a) and 4.46 (1H, m, H-23b); δ_C 37.6 (C-20), 176.7 (C-21), 34.1 (C-22) and 72.6 (C-23)]. The 1H - 1H COSY spectrum of compound 1 showed correlations in H_1 - H_2 , H_6 - H_7 , H_9 - H_{10} - H_{11} , and H_{15} - H_{16} - H_{17} - H_{20} - H_{22} - H_{23} , supporting the presence of an azadirone-type limonoid structure with acetoxyl group and 3-substituted- γ -lactone ring. The HMBC correlations from the tertiary methyl protons to their neighboring carbons, enabled the assignment of the six singlet methyls in C-4 ($2 \times CH_3$), C-23, C-10, C-13 and C-2' respectively. Furthermore, the olefinic protons at δ_H 5.85 (H-2; d, $J = 10.7$ Hz), and 7.13 (H-1; d, $J = 10.7$ Hz) correlated to the carbonyl signal at δ_C 204.6 (C-3), indicating an unsubstituted enone in the A ring, similar to azadirone (Lavie et al., 1971). Correlation from oxygenated methylene protons at δ_H 3.92 (H-23), 4.46

(H-23) and 1.70 (H-17) to carbon at δ_C 37.6 (C-20) and carbonyl lactone 176.5 (C-21), were used to assign carbonyl lactone which was located on C-21. Similarly, an oxygenated methine proton at δ_H 5.23 (H-7) and correlations for H-7 and H-14 of C-1' (δ_C 170.1), indicated the presence of acetoxyl group at C-7. The relative configuration of compound 1 was determined by a NOESY experiment (Fig. 3), which showed NOE correlations between CH_3 -30 and H-7, indicated that the acetyl group at C-7 is α -oriented. Similar to the observations from NOESY, showed the cross peak between CH_3 -18 and H-20, indicated that H-20 was α -oriented. Furthermore, the NOESY cross peak also observed between CH_3 -30/H-17, indicated that the β -pyrone ring at C-17 was α -oriented. Therefore, the structure of compound 1 was elucidated as the new azadirone-type limonoid and namely as pentandricine B.

The pentandricine C (2) was isolated as a colorless amorphous solid, with the molecular formula established as $C_{26}H_{36}O_4$ from HR-TOFMS m/z 412.2672 $[M+H]^+$ and NMR data (Table 1), thus having 9 index of hydrogen deficiency. Its UV spectrum showed an absorption maximum at 227 nm ($\log \epsilon$ 4.1), indicating the presence of an enone group as in compound 1. The IR spectrum showed bands which were ascribed to hydroxy (ν_{max} 3530 cm^{-1}), olefinic (ν_{max} 3102 cm^{-1}), aliphatics (ν_{max} 2861 cm^{-1}), carbonyl lactone (ν_{max} 1775 cm^{-1}) and an α,β -unsaturated carbonyl (ν_{max} 1665 cm^{-1}). The NMR spectra of 2 was very similar to those of 1, except with the absence of acetyl group at δ_H [1.94 (3H, s); δ_C 21.2, 170.1] and the presence of an oxygenated methine at δ_H [3.78 (1H, t, $J = 2.4$ Hz); δ_C 72.4], indicating that 2 was a deacetyl derivative of 1. In the HMBC spectrum, an oxygenated methine at δ_H 3.78 was correlated to δ_C 23.8 (C-5) and δ_C 42.8 (C-8), suggesting that a new secondary hydroxyl was located at C-7. The relative configuration was determined from NOESY spectrum (Fig. 3). Therefore, leading to the structure of 2 had been elucidated as the azadirone-type limonoid, namely as pentandricine C.

Pentandricine D (3), colorless amorphous solid, has the molecular formula $C_{30}H_{40}O_7$ the HR-TOFMS spectra m/z 514.1892 $[M+H]^+$ and NMR data (Table 1) with 11 index of hydrogen deficiency. The UV spectrum (λ_{max} 235 nm, $\log \epsilon$ 4.3), indicating the presence of an enone group. The IR spectrum showed bands which were assigned to an olefinic (ν_{max} 3017 cm^{-1}), aliphatic (ν_{max} 2944 cm^{-1}), lactone (ν_{max} 1775 cm^{-1}), conjugated ester (ν_{max} 1722 cm^{-1}) and an α,β -unsaturated carbonyl (ν_{max} 1665 cm^{-1}) groups. The NMR spectra of 3 was similar to those of 1, (Table 2) except for the replacement of the methylene signals at δ_H 2.50 (1H, dd, $J = 8.0$, 17.4 Hz), 2.30 (1H, dd, $J = 5.6$, 8.0 Hz); δ_C 46.2] in compound 1 signals for the acetyl group at δ_H 1.97 (3H, s); δ_C 21.2, 170.1, indicating that 3 is acetoxyl (δ_C 72.9; δ_H 5.22) derivative of 1. In the HMBC spectrum, a methyl signal at δ_H 1.97 and oxygenated methine at δ_H 5.22 was correlated to δ_C 170.0 (C-1'), suggesting the additional acetyl group was attached at C-6. Consequently, the structure of 3 was elucidated as new azadirone-type limonoid which was given the trivial name pentandricine D.

The known compounds 6 α -acetoxyl-14 β ,15 β -epoxyazadirone (4) (Chatterjee et al., 1989; Mulholland et al., 1994; Neto et al., 1995), paniculatin (5) (Bordoloi et al., 1993), 14 β ,15 β -epoxynimonol (6) (Govindachari et al., 1989) and nimonol (7) (Suresh et al., 1997; Siddiqui et al., 2004) were identified by comparison of their spectroscopic data with reported values.

Compounds 1–7 were evaluated for their cytotoxicity against MCF-7 breast cancer cell line (Table 2) according to a method described previously (Skehan et al., 1990; Supriatno et al., 2018; Hadisaputri et al., 2012). Nimonol (7) showed the strongest activity against MCF-7, suggesting that the presence of furan ring and acetyl group plays important role for the cytotoxic activity of limonoid structure. These results are in accordance with previous studies, where the furan and acetyl groups play an important role in the cytotoxic activity of the limonoid structures (Supriatno et al., 2018; Mohamad et al., 2009; Maneerat et al., 2008).

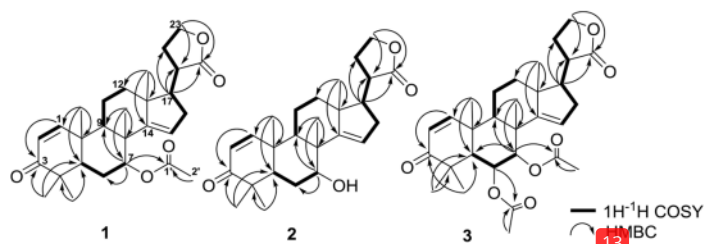


Fig. 2. Selected HMBC and COSY correlations for pentandricine B–D (1–3).

Table 1

NMR data of compounds 1–3 (600 MHz for ^1H and 150 MHz for ^{13}C).

Position	1	2	3			
	¹³ C-NMR δ _C (mult.)	¹ H-NMR δ _C [(ΣH, mult, J(Hz))]	¹³ C-NMR δ _C (ppm)	¹ H-NMR δ _C [(ΣH, mult, J(Hz))]	¹³ C-NMR δ _C (mult.)	¹ H-NMR δ _C [(ΣH, mult, J(Hz))]
1	157.9 (d)	7.13 (1H, d, 10.8)	157.9 (d)	7.13 (d, 10.8)	157.7 (d)	7.13 (1H, d, 10.8)
2	125.7 (d)	5.85 (1H, d, 10.8)	125.7 (d)	5.85 (d, 10.8)	126.3 (d)	5.85 (1H, d, 10.8)
3	204.6 (s)	—	204.3 (s)	—	204.8 (s)	—
4	40.0 (s)	—	39.9 (s)	—	39.9 (s)	—
5	46.2 (d)	2.50 (1H, dd, 8.0, 17.4)	46.2 (d)	2.50 (1H, dd, 8.0, 17.4)	46.2 (d)	2.50 (1H, dd, 8.0, 17.4)
6	23.8 (d)	1.77 (1H, m)	22.8 (d)	1.77 (1H, m)	72.9 (d)	5.22 (1H, m)
7	74.4 (d)	5.23 (1H, t, 2.4)	72.4 (d)	3.78 (1H, t, 2.4)	73.6 (d)	5.23 (1H, t, 2.4)
8	42.8 (s)	—	42.8 (s)	—	42.8 (s)	—
9	38.4 (d)	2.20 (1H, m)	38.4 (d)	2.20 (1H, m)	38.4 (d)	2.20 (1H, m)
10	44.2 (s)	—	44.2 (s)	—	44.2 (s)	—
11	16.5 (t)	1.93 (1H, m)	16.5 (t)	1.93 (1H, m)	16.5 (d)	1.93 (1H, m)
12	33.8 (t)	1.75 (1H, m)	33.8 (t)	1.72 (1H, m)	—	1.74 (1H, m)
13	46.7 (s)	1.54 (1H, m)	46.7 (s)	1.54 (1H, m)	—	1.54 (1H, m)
14	158.8 (s)	1.73 (1H, m)	158.8 (s)	1.73 (1H, m)	—	1.73 (1H, m)
15	119.0 (d)	—	118.9 (d)	—	158.9 (s)	—
16	34.9 (t)	5.28 (8, dd, 1.8, 3.6)	34.9 (t)	5.30 (1H, dd, 1.8, 3.6)	118.0 (d)	5.28 (8, dd, 1.8, 3.6)
17	58.2 (d)	2.04 (1H, m)	58.2 (19)	2.04 (1H, m)	—	2.04 (1H, m)
18	20.0 (q)	2.14 (1H, dd, 3.6, 7.8)	20.0 (q)	2.14 (1H, dd, 3.6, 7.8)	57.3 (d)	2.14 (1H, dd, 3.6, 7.8)
19	19.1 (q)	1.70 (1H, dt, 3.0, 10.2)	19.1 (q)	1.70 (1H, dt, 3.0, 10.2)	20.0 (q)	1.70 (1H, dt, 3.0, 10.2)
20	37.6 (d)	1.00 (7, s)	37.6 (d)	1.01 (3H, s)	19.1 (q)	1.00 (7, s)
21	176.7 (s)	1.16 (3H, s)	176.4 (s)	1.17 (3H, s)	37.6 (d)	1.16 (3H, s)
22	34.1 (t)	2.70 (1H, m)	34.1 (t)	2.70 (1H, m)	176.2 (s)	2.70 (1H, m)
23	72.5 (t)	2.17 (1H, m)	72.5 (19)	2.17 (1H, m)	—	2.17 (1H, m)
28	21.4 (q)	2.20 (1H, m)	21.4 (q)	2.17 (1H, m)	34.1 (t)	2.16 (1H, m)
29	27.1 (q)	3.92 (7, m)	27.1 (q)	3.92 (1H, m)	—	3.92 (7, m)
30	27.5 (q)	4.46 (1H, m)	27.5 (q)	4.46 (1H, m)	70.2 (t)	4.46 (1H, m)
1'	170.1 (s)	1.06 (3H, s)	170.1 (s)	1.07 (3H, s)	—	1.06 (3H, s)
2'	21.2 (q)	1.06 (3H, s)	21.2 (q)	1.07 (3H, s)	21.1 (q)	1.06 (3H, s)
3'	—	1.16 (3H, s)	—	1.17 (3H, s)	170.0 (s)	1.16 (3H, s)
4'	—	1.94 (3H, s)	—	—	21.2 (q)	1.91 (3H, s)
5'	—	—	—	—	—	—
6'	—	—	—	—	—	—
7'	—	—	—	—	—	—
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60'	—	—	—	—	—	—

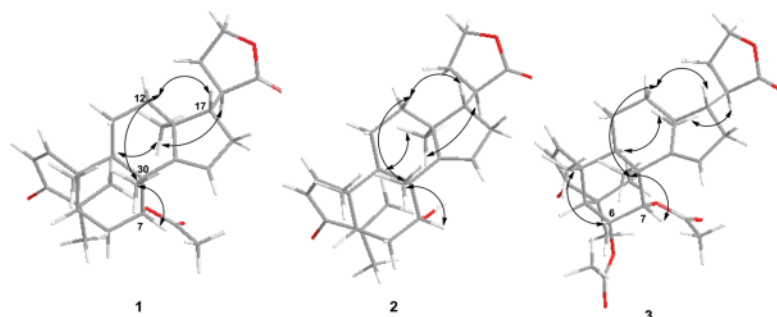


Fig. 3. Selected NOESY correlations for pentandricine B–D (1–3).

Table 2

Cytotoxicity of compounds 1–7 against MCF-7 breast cancer cell line.

Compounds	IC ₅₀ (μM)
Pentandricine B (1)	212.02 ± 0.015
Pentandricine C (2)	122.02 ± 0.006
Pentandricine D (3)	313.92 ± 0.015
6α-acetoxy-14β,15β-epoxyazadirone (4)	93.13 ± 0.011
Paniculatin (5)	64.88 ± 0.015
14β,15β-epoxynimonol (6)	32.22 ± 0.025
Nimonol (7)	22.03 ± 0.026
Cisplatin* (Chavoshi et al., 2017)	3.20 ± 0.006

* positive control.

3. Experimental procedures

3.1. General

UV spectra was measured using a TECAN Infinite M200 pro, with MeOH. The IR spectra and mass spectra were recorded on a SHIMADZU IR Prestige-21 in KBr and Waters Xevo QTOF MS respectively. Using a JEOL ECZ-600, the NMR data was recorded at 600 MHz for ¹H and 150 MHz for ¹³C, using TMS as internal standard. Column chromatography was conducted on the silica gel 60 (70–230 and 230–400 mesh, Merck), after which TLC analysis was carried out on 60 GF₂₅₄ (Merck, 0.25 mm) using various solvent systems, in order to detect spots by irradiating under ultraviolet-visible light (257 and 364 nm) and heating of silica gel plates, sprayed with 75 % vanillin sulfate in ethanol (v/v = 75:25) and Ehrlich's reagents.

3.2. Plant material

The stem bark of *C. pentandrus* were collected from Bogor Botanical Garden, Bogor, West Java Province, Indonesia in June 2016. Furthermore, the plant was identified by Mr. Ismail, a staff of the Bogoriense Herbarium, Bogor, Indonesia. Finally, a voucher specimen (No. Bo-104) was deposited at the Herbarium.

3.3. Extraction and isolation

The dried ground stem bark (1.8 kg) of *C. pentandrus* was extracted with methanol (14 L), at room temperature for 7 days. After removal of the solvent under vacuum, the viscous concentrated MeOH extract (340.01 g) was suspended in H₂O and then successively partitioned with *n*-hexane, EtOAc and *n*-butanol. The *n*-hexane extract (10.90 g) was fractionated by column chromatography on silica gel, using a gradient of *n*-hexane, EtOAc and MeOH (5:1:5:3.5) stepwise resulting into eight fractions (A–H). Fraction D (725.1 mg) was subjected to column chromatography on silica gel using *n*-hexane–CHCl₃–EtOAc (5 % stepwise), as eluting solvent to afford seven subfractions (D1–D7). Subfraction D3 (80.1 mg) was chromatographed on a column of silica gel, eluted with *n*-hexane: CHCl₃: EtOAc (5:1.5:3.5), to give seven subfractions (D3A–D3G). Similarly, subfraction D3B (20.3 mg) was chromatographed on preparative TLC on silica gel GF₂₅₄, eluted with *n*-hexane: CHCl₃: EtOAc (4:2.5:3.5) and 0.1 mL of formic acid, to give 1 (6 mg) and 2 (3.6 mg). In the same way, subfraction D5 (120.3 mg) was chromatographed on silica gel eluted with *n*-hexane: CHCl₃: EtOAc (5:1.5:3.5) and 0.1 mL of formic acid, to give seven subfractions (D5A–D5G). Similarly, subfraction D5D (60.2 mg) was chromatographed on silica gel, eluted with *n*-hexane:CHCl₃:EtOAc (3:3.5:3.5) and 0.4 mL of formic acid to give 3 (18.3 mg), while fraction F (813.3 mg) was subjected to column chromatography on silica gel using a mixture of *n*-hexane–CHCl₃–EtOAc (5 % stepwise), as eluting solvents to afford six subfractions (F1–F6). Subfraction F2 (120.4 mg) was chromatographed on silica gel, eluted with CHCl₃: EtOAc (7:3) and 0.1 mL of formic acid to give five subfractions (F2A–F2E). Furthermore,

subfraction F2B (25 mg) was chromatographed on silica gel with *n*-hexane:CHCl₃:EtOAc (22:1) to give 4 (6.3 mg) and 5 (5.9 mg), while fraction G (518.2 mg) was subjected to column chromatography on silica gel, eluted with a gradient of *n*-hexane–CHCl₃–EtOAc (5 % stepwise) to give six subfractions (G1–G8). Lastly, subfraction G6 (111.3 mg) was column chromatographed on silica gel, eluted with CHCl₃:EtOAc:MeOH (7:2.5:0.5), to give five subfractions (F2A–F2E), while subfraction F2B (34 mg) was column chromatographed on silica gel, eluted with CHCl₃:EtOAc:MeOH (7:2.5:0.5) to give 6 (8.8 mg) and 7 (5.8 mg).

3.3.1. Pentandricine B (1)

Colorless amorphous solid; [α]_D²⁰ + 24° (c 0.12, MeOH); UV (MeOH) λ_{max} 240 nm (log ε 4.2); IR (KBr) ν_{max} 3017, 2944, 1775, 1722 and 1665 cm^{−1}; HR-TOFMS *m/z* 454.2871 [M+H]⁺, (calcd. C₂₈H₃₈O₅ *m/z* 454.2828); ¹H-NMR (CDCl₃, 600 MHz), see Table 1; ¹³C-NMR (CDCl₃, 150 MHz), see Table 1.

3.3.2. Pentandricine C (2)

Colorless amorphous solid; [α]_D²⁰ + 23° (c 0.13, MeOH); UV (MeOH) λ_{max} 227 nm (log ε 4.1); IR (KBr) ν_{max} 3530, 3102, 2861, 1775 and 1665 cm^{−1}; HR-TOFMS *m/z* 412.2672 [M+H]⁺, (calcd. C₂₆H₃₆O₄ *m/z* 412.2622); ¹H-NMR (CDCl₃, 600 MHz), see Table 1; ¹³C-NMR (CDCl₃, 150 MHz), see Table 1.

3.3.3. Pentandricine D (3)

Colorless amorphous solid; [α]_D²⁰ + 24° (c 0.13, MeOH); UV (MeOH) λ_{max} 235 nm (log ε 4.3); IR (KBr) ν_{max} 3017, 2944, 1775, 1722 and 1665 cm^{−1}; HR-TOFMS *m/z* 514.1892 [M+H]⁺, (calcd. C₃₀H₄₀O₇ *m/z* 514.1822); ¹H-NMR (CDCl₃, 600 MHz), see Table 1; ¹³C-NMR (CDCl₃, 150 MHz), see Table 1.

3.4. Bioassays for cytotoxic activity

The MCF-7 cells were seeded into 96-well plates with an initial cell density of approximately 3 × 10⁴ cells cm^{−3}, after which the cell was incubated for attachment and growth, alongside additions of varying sample concentrations. The compounds added were first dissolved in DMSO at the required concentration, subsequently preparing six desirable concentrations using PBS (phosphoric buffer solution, pH = 7.30–7.65). Control wells received only DMSO, as the assay was terminated after a 48 h incubation period by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named as thiazol blue], after which the incubation was continued for another 4 h. Later on, the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added, after which it was left for another 24 h incubation period. Optical density was read using a micro plate reader at 550 nm, with recorded IC₅₀ values from the plotted graph of percentage live cells, were compared to control (%) receiving only PBS and DMSO, versus the tested concentration of compounds (μM). Noteworthy, the IC₅₀ value is the concentration required for 50 % growth inhibition, as each assay and analysis were run in triplicate and averaged.

3.4. Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.phytol.2019.11.002>.

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