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^{1}H AND ^{13}C NMR ASSIGNMENTS OF PHENOLIC AND OTHER CONSTITUENTS FROM *MILIUSA SINENSIS* LEAVES AND THEIR CYTOTOXIC ACTIVITY

Cao Thi Hue[1], Pham Thanh Binh[2], Luc Quang Tan[3], Hoang Van Hung[3], Vu Thanh Trung[2], Nguyen Thi Lan Huong[1], Nguyen Manh Ha[4], Nguyen Van Thanh[2], Nguyen Phuong Thao[2,*]

[1]*Thuyloi University, Hanoi, Vietnam;*

[2]*Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam;*

[3]*Thai Nguyen University-Lao Cai Campus, Lao Cai Province, Vietnam;*

[4]*University of Mining and Geology, Hanoi, Vietnam*

*Corresponding author: thaonp@imbc.vast.vn

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Summary

^{1}H and ^{13}C NMR Assignments of Phenolic and other Constituents from *Miliusa sinensis* Leaves and their Cytotoxic Activity

Phytochemical investigation of a methanolic extract of *Miliusa sinensis* leaves led to the isolation of eight compounds (**1–8**), using various chromatographic separations. Their structures were elucidated to be pteleifoside C (**1**), melisimplexin (**2**), 3,5,6,7,3',4'-hexamethoxyflavone (**3**), 6'-*O*-*β*-D-apiofuranosylthalictoside (**4**), osmanthuside H (**5**), icariside D_1 (**6**), 3,4-dimethoxyphenol 1-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**7**), and 3,4,5-trimethoxyphenol 1-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**8**) by detailed analysis via spectroscopic techniques (1D, 2D NMR, and MS data) as well as comparison with those reported. All of the isolates were evaluated for their cytotoxicity against HepG2, DU145, A549, and MCF7 cancer cell lines, using the MTT assay. Compounds **1-8** showed weak or fewer effects against the tested cancer cell lines. This is the first time that glycoside constituents from *M. sinensis* leaves and their cytotoxic activity were reported.

Keywords: *Miliusa sinensis*; *Annonaceae*; *Flavonoid*; *Megastigmane*; *Phenolic*; *Cytotoxic activity*.

1. Introduction

Miliusa is a small genus in the Annonaceae family, comprising about 60 known species of shrubs and trees distributed in the tropical rainforests of many Asian countries, such as China, Thailand, and Vietnam [1]. The genus *Miliusa* has only nine species distributed from the North to the South of Vietnam [2]. *Miliusa sinensis* Finet and Gagnep. (syn. *Euodia lyi* H. Lév.), an evergreen tree up to 6 m tall, is distributed in the forest regions of northern mainland Vietnam [2],[3]. In our country, previous studies of this plant have investigated its chemical properties including miliusanes [4] and flavonoid constituents [5],[6]. Some miliusanes have exhibited unique response patterns against a panel of cancer cell lines comprising lung, colon, prostate, breast, cervical, and leukemia cell lines [5],[7]. In work of this type that has been conducted to date, most previous studies were mainly focused on the constituents for the less polar fractions [4],[6]. However, little is known about the water-soluble chemical profiles despite water decoction being the main ingredient. For this reason, our investigation focused on the water layer constituents of *M. sinensis* leaves.

In the present study, the isolation and structural elucidation of eight compounds (**1-8**, Fig. 1) as well as their cytotoxicity toward the tumor cells HepG2, DU145, A549, and MCF7, are discussed.

2. Material and methods

2.1. Plant material

The leaves of *Miliusa sinensis* Finet and Gagnep. were collected at Na Hang, Tuyen Quang province, Vietnam in March 2020, and taxonomically identified by Dr. Nguyen The Cuong (Institute of Ecology and Biological Resources, VAST). A voucher specimen (NCCB-01-MS) was deposited at the Herbarium of the Institute of Marine Biochemistry and Biotechnology Department, Thuyloi University.

2.2. Chemicals and equipments

Optical rotations were determined on a JASCO P-2000 polarimeter. The NMR spectra were recorded on an AVANCE III HD 500 spectrometer with TMS used as an internal standard. The HR-ESI-MS were acquired on an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. MPLC was carried out on a Biotage-

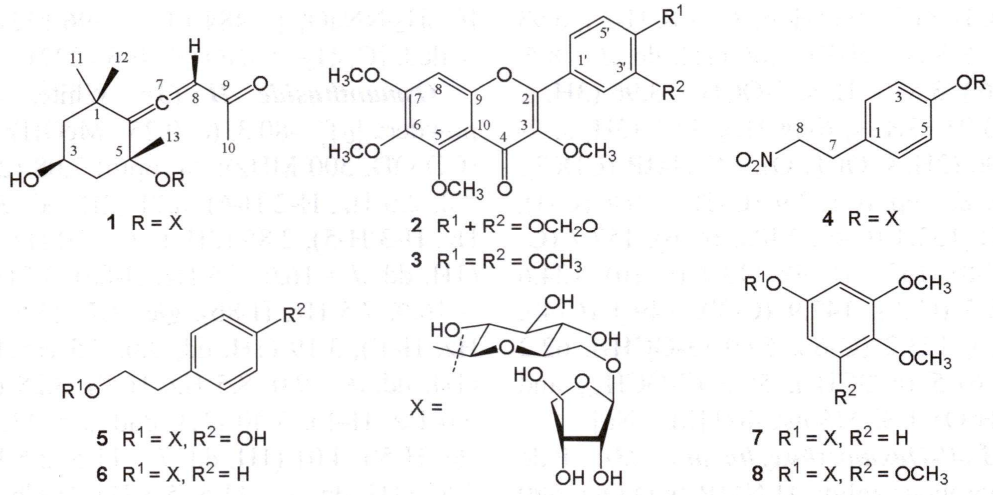

Fig. 1. Structures of compounds **1–8**

Isolera One system. Column chromatography was conducted on *silica gel*, Diaion HP-20, Sephadex LH-20, and RP-18. Analytical thin-layer chromatography systems were performed on precoated *silica gel* 60 F_{254} and RP-18 F_{254S} plates, and the isolated compounds were visualized by spraying with 10% H_2SO_4 in water and then heating for 1.5-2 minutes.

2.3. Extraction and isolation

The leaves of *M. sinensis* (2.5 kg) were dried, ground, and extracted with 95% aqueous MeOH (15 L) three-time (each 1 day) at room temperature under reflux and filtered. The filtrate was combined and concentrated under reduced pressure using a rotavapor to obtain a MeOH extract (150 g), which was suspended in distilled water (1.5 L) and then successively solvent partitioned with *n*-hexane, dichloromethane (3 times each) to yield *n*-hexane (23 g), dichloromethane (30 g), and a water layer (W). By comparison with a TLC analysis of the three fractions resulted from the solvent partitioning indicated the presence of metabolites in the water layer.

The water layer was applied to a Diaion HP-20 column and eluted with stepwise additions of MeOH in water (0%, 25%, 50%, 75%, 100%) to obtain four major subfractions (W.1 to W.4). In view of the identical TLC profiles, fraction W-2 (19 g) was successively separated on *silica gel* MPLC (column: Biotage SNAP Cartridge, KP-SIL, 100 g) using a mobile phase of CH_2Cl_2-MeOH (0-5 min 25% MeOH, 6-50 min 25-50% MeOH, 56-80 min 90% MeOH, 81-95 min 100% MeOH, 15 mL/min, 95 min) to obtain six subfractions (W-2.1 to W-2.6). Further purification of subfraction W-2.2 (0.7 g) was subjected to Sephadex LH-20 open CC with a solvent system MeOH-H_2O (1:1, v/v) and a *silica gel* CC, eluting with CH_2Cl_2-MeOH (20:1), resulting in the purification of **4** (6.8 mg) and **8** (5.2 mg). Fraction W-2.4 (5.8 g) was further applied to a *silica gel* column using gradient mixtures of CH_2Cl_2-MeOH-H_2O (6:1:0.1, v/v) to yield four subfractions (W-2.4a to W-2.4d). One of these fractions, subfraction W-2.4a (0.8 g) was successively applied to an open RP-18 column and further subjected to a Sephadex LH-20 chromatography eluting with MeOH-H_2O (1:4, v/v) as mobile phase to yield **2** (4.1 mg), **3** (3.2 mg), and **7** (6.5 mg). Additionally, subfraction W-2.4b (1.05 g) was rechromatographed on *silica gel* by MPLC using a MeOH-EtOAc gradient system (20-100%) to give three subfractions (W-2.4b1 to W-2.4b3). Compound **1** (5.5 mg) was further obtained from subfraction W-2.4b1 by an open RP-18 column eluted with MeOH-H_2O (1:2.5, v/v). Finally, subfraction W-2.4b3 (0.17 g) was further purified by a Sephadex LH-20 column with MeOH solvent and a *silica gel* CC with CH_2Cl_2-EtOAc (4:1, v/v) as the mobile phase, resulting in the isolation of compounds **5** (4.5 mg) and **6** (6.5 mg).

Pteleifoside C (1): White, amorphous powder; $[\alpha]_D^{24}$ -122.3 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3416, 2928, 1940, 1665, 1457, 1365, 1245, 1071, and 818 cm^{-1}; ^1H (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Table 1; HR-ESI-MS *m/z* 541.2270 $[M + Na]^+$ (calcd. $[C_{24}H_{38}NaO_{12}]^+$, 541.2266).

Melisimplexin (2): Pale yellow, amorphous solid; ^1H NMR ($CDCl_3$, 500 MHz): δ_H (ppm)

6.73 (1H, s, H-8), 7.59 (1H, d, J = 1.8, H-2'), 6.93 (1H, d, J = 8.5 Hz, H-5'), 7.66 (1H, dd, J = 8.5, 1.8 Hz, H-6'), 3.87 (3H, s, 3-OC$\underline{H}_3$), 3.96 (3H, s, 5-OC$\underline{H}_3$), 3.91 (3H, s, 6-OC$\underline{H}_3$), 4.01 (3H, s, 7-OC$\underline{H}_3$), 6.06 (2H, s, OC$\underline{H}_2$O); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C (ppm) 152.9 (C-2), 140.8 (C-3), 173.6 (C-4), 152.4 (C-5), 140.2 (C-6), 157.7 (C-7), 96.0 (C-8), 153.5 (C-9), 113.1 (C-10), 124.6 (C-1'), 108.5 (C-2'), 147.9 (C-3'), 149.4 (C-4'), 108.4 (C-5'), 123.2 (C-6'), 59.9 (3-O$\underline{C}H_3$), 62.2 (5-O$\underline{C}H_3$), 61.5 (6-O$\underline{C}H_3$), 56.3 (7-O$\underline{C}H_3$), and 101.6 (O$\underline{C}H_2$O); ESI-MS m/z 409 $[M + Na]^+$.

3,5,6,7,3',4'-Hexamethoxyflavone (3): Pale yellow, amorphous solid; ^{1}H NMR (CD_3OD, 500 MHz): δ_H (ppm) 7.03 (1H, s, H-8), 7.73 (1H, d, J = 1.8, H-2'), 7.09 (1H, d, J = 8.5 Hz, H-5'), 7.76 (1H, dd, J = 8.5, 1.8 Hz, H-6'), 3.80 (3H, s, 3-OC$\underline{H}_3$), 3.95 (3H, s, 5-OC$\underline{H}_3$), 3.98 (3H, s, 6-OC$\underline{H}_3$), 4.01 (3H, s, 7-OC$\underline{H}_3$), 3.92 (3H, s, 3'-OC$\underline{H}_3$), 3.93 (3H, s, 4'-OC$\underline{H}_3$); ^{13}C NMR (CD_3OD, 125 MHz): δ_C (ppm) 155.1 (C-2), 141.8 (C-3), 177.4 (C-4), 153.1 (C-5), 141.5 (C-6), 159.8 (C-7), 97.7 (C-8), 155.5 (C-9), 113.5 (C-10), 124.2 (C-1'), 112.8 (C-2'), 150.3 (C-3'), 152.9 (C-4'), 112.5 (C-5'), 123.3 (C-6'), 60.3 (3-O$\underline{C}H_3$), 62.6 (5-O$\underline{C}H_3$), 61.8 (6-O$\underline{C}H_3$), 57.0 (7-O$\underline{C}H_3$), 56.6 (3'-O$\underline{C}H_3$), and 54.4 (4'-O$\underline{C}H_3$); ESI-MS m/z 425 $[M + Na]^+$.

6'-O-β-D-Apiofuranosylthalictoside (4): White, amorphous powder; $[\alpha]_D^{24}$ -108.5 (c 0.15, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ_H (ppm) 7.20 (2H, br d, J = 8.5 Hz, H-2/H-6), 7.07 (2H, br d, J = 8.5 Hz, H-3/H-5), 3.25 (2H, t, J = 7.0 Hz, H-7), 4.69 (2H, t, J = 7.0 Hz, H-8), **glc**: 4.85 (1H, d, J = 7.5 Hz, H-1'), 3.46 (1H, overlapped signal, H-2'), 3.43 (1H, overlapped signal, H-3'), 3.35 (1H, t, J = 9.0 Hz, H-4'), 3.60 (1H, m, H-5'), 4.03 (1H, dd, J = 11.5, 2.0 Hz, H-6'a), 3.64 (1H, dd, J = 11.5, 5.0 Hz, H-6'b), **api**: 4.99 (1H, d, J = 2.5 Hz, H-1"), 3.92 (1H, d, J = 2.5 Hz, H-2"), 3.99 (1H, d, J = 10.0 Hz, H-4"a), 3.77 (1H, d, J = 10.0 Hz, H-4"b), and 3.58 (2H, overlapped signals, H-5"); ^{13}C NMR (CD_3OD, 125 MHz): δ_C (ppm) 131.7 (C-1), 130.7 (C-2/C-6), 118.1 (C-3/C-5), 158.2 (C-4), 33.5 (C-7), 77.5 (C-8), **glc**: 102.4 (C-1'), 75.0 (C-2'), 77.9 (C-3'), 71.6 (C-4'), 77.0 (C-5'), 68.8 (C-6'), **api**: 111.0 (C-1"), 78.0 (C-2"), 80.4 (C-3"), 74.9 (C-4"), and 65.5 (C-5"); HR-ESI-MS m/z 484.1440 $[M + Na]^+$ (calcd. $[C_{19}H_{27}NNaO_{12}]^+$, 484.1431), 496.1221 $[M + Cl]^-$ (calcd. $[C_{19}H_{27}{}^{35}ClNO_{12}]^-$, 496.1222).

Osmanthuside H (5): White, amorphous powder; $[\alpha]_D^{24}$ -80.3 (c 0.25, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ_H (ppm) 7.08 (2H, dd, J = 8.0, 2.0 Hz, H-2/H-6), 6.71 (2H, dd, J = 8.0, 2.0 Hz, H-3/H-5), 2.85 (2H, t, J = 7.0 Hz, H-7), 4.02 (1H, dd, J = 16.0, 7.5 Hz, H-8a), 3.71 (1H, dd, J = 16.0, 7.5 Hz, H-8b), **glc**: 4.77 (1H, d, J = 7.5 Hz, H-1'), 3.19 (1H, dd, 9.0, 7.5 Hz, H-2'), 3.36 (1H, dd, J = 9.0, 8.5 Hz, H-3'), 3.28 (1H, t, J = 9.0 Hz, H-4'), 3.40 (1H, ddd, J = 11.5, 9.0, 2.5 Hz, H-5'), 4.01 (1H, dd, J = 11.5, 2.5 Hz, H-6'a), 3.62 (1H, dd, J = 11.5, 5.5 Hz, H-6'b), **api**: 5.02 (1H, d, J = 2.5 Hz, H-1"), 3.91 (1H, d, J = 2.5 Hz, H-2"), 3.98 (1H, d, J = 10.0 Hz, H-4"a), 3.77 (1H, d, J = 10.0 Hz, H-4"b), and 3.58 (2H, br s, H-5"); ^{13}C NMR (CD_3OD, 125 MHz): δ_C (ppm) 130.8 (C-1), 130.9 (C-2/C-6), 116.2 (C-3/C-5), 156.8 (C-4), 36.4 (C-7), 72.2 (C-8), **glc**: 104.4 (C-1'), 75.1 (C-2'), 78.1 (C-3'), 71.7 (C-4'), 76.9 (C-5'), 68.7 (C-6'), **api**: 110.9 (C-1"), 78.0 (C-2"), 80.5 (C-3"), 75.0 (C-4"), and 65.5 (C-5"); ESI-MS m/z 433 $[M + H]^+$, 455 $[M + Na]^+$.

Icariside D_1 (6): Colorless syrup; $[\alpha]_D^{24}$ -73.7 (c 0.15, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ_H (ppm) 7.27 (2H, m, H-2/H-6), 7.28 (2H, m, H-3/H-5), 7.18 (1H, m, H-4), 2.96 (2H, t, J = 7.0 Hz, H-7), 4.06 (1H, dd, J = 16.0, 7.5 Hz, H-8a), 3.79 (1H, dd, J = 16.0, 7.5 Hz, H-8b), **glc**: 4.31 (1H, d, J = 7.5 Hz, H-1'), 3.20 (1H, dd, 9.0, 7.5 Hz, H-2'), 3.35 (1H, dd, J = 9.0, 8.5 Hz, H-3'), 3.29 (1H, t, J = 9.0 Hz, H-4'), 3.42 (1H, ddd, J = 11.5, 9.0, 2.5 Hz, H-5'), 4.01 (1H, dd, J = 11.5, 2.5 Hz, H-6'a), 3.63 (1H, dd, J = 11.5, 5.5 Hz, H-6'b), **api**: 5.02 (1H, d, J = 2.5 Hz, H-1"), 3.91 (1H, d, J = 2.5 Hz, H-2"), 3.98 (1H, d, J = 10.0 Hz, H-4"a), 3.77 (1H, d, J = 10.0 Hz, H-4"b), and 3.58 (2H, br s, H-5"); ^{13}C NMR (CD_3OD, 125 MHz): δ_C (ppm) 140.0 (C-1), 129.3 (C-2/C-6), 130.0 (C-3/C-5), 127.2 (C-4), 37.2 (C-7), 71.8 (C-8), **glc**: 104.4 (C-1'), 75.1 (C-2'), 78.0 (C-3'), 71.7 (C-4'), 76.9 (C-5'), 68.7 (C-6'), **api**: 110.9 (C-1"), 78.0 (C-2"), 80.5 (C-3"), 75.0 (C-4"), and 65.6 (C-5"); ESI-MS m/z 439 $[M + Na]^+$.

3,4-Dimethoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (7): White, amorphous powder; $[\alpha]_D^{24}$ -110.9 (c 0.15, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ_H (ppm)

6.80 (1H, d, J = 2.5 Hz, H-2), 6.89 (1H, d, J = 8.5 Hz, H-5), 6.70 (1H, dd, J = 8.5, 2.5 Hz, H-6), 3.83 (3H, s, 3-OC$\underline{H}_3$), 3.80 (3H, s, 4-OC$\underline{H}_3$), **glc**: 4.77 (1H, d, J = 7.5 Hz, H-1'), 3.44 (1H, dd, 9.0, 7.5 Hz, H-2'), 3.35 (1H, overlapped signal, H-3'), 3.45 (1H, overlapped signal, H-4'), 3.56 (1H, m, H-5'), 4.04 (1H, dd, J = 11.5, 2.5 Hz, H-6'a), 3.63 (1H, dd, J = 11.5, 5.5 Hz, H-6'b), **api**: 5.00 (1H, d, J = 2.5 Hz, H-1"), 3.92 (1H, d, J = 2.5 Hz, H-2"), 3.98 (1H, d, J = 10.0 Hz, H-4"a), 3.77 (1H, d, J = 10.0 Hz, H-4"b), and 3.59 (2H, br s, H-5"); ^{13}C NMR (CD_3OD, 125 MHz): δ_C (ppm) 153.8 (C-1), 104.3 (C-2), 151.1 (C-3), 146.2 (C-4), 114.1 (C-5), 109.4 (C-6), 56.6 (3-O$\underline{C}H_3$), 57.2 (4-O$\underline{C}H_3$), **glc**: 103.4 (C-1'), 75.0 (C-2'), 78.0 (C-3'), 71.7 (C-4'), 77.0 (C-5'), 68.8 (C-6'), **api**: 110.9 (C-1"), 77.9 (C-2"), 80.5 (C-3"), 75.0 (C-4"), and 65.5 (C-5"); ESI-MS m/z 471 $[M + Na]^+$.

3,4,5-Trimethoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (8): White, amorphous powder; $[\alpha]_D^{24}$ -93.4 (c 0.15, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ_H (ppm) 6.48 (2H, s, H-2/H-6), 3.83 (6H, s, 3,5-OC$\underline{H}_3$), 3.72 (3H, s, 4-OC$\underline{H}_3$), **glc**: 4.81 (1H, d, J = 7.5 Hz, H-1'), 3.44 (1H, dd, 9.0, 7.5 Hz, H-2'), 3.35 (1H, overlapped signal, H-3'), 3.45 (1H, overlapped signal, H-4'), 3.56 (1H, m, H-5'), 4.06 (1H, dd, J = 11.5, 2.5 Hz, H-6'a), 3.62 (1H, dd, J = 11.5, 5.5 Hz, H-6'b), **api**: 4.98 (1H, d, J = 2.5 Hz, H-1"), 3.92 (1H, d, J = 2.5 Hz, H-2"), 3.97 (1H, d, J = 10.0 Hz, H-4"a), 3.77 (1H, d, J = 10.0 Hz, H-4"b), and 3.57 (2H, br s, H-5"); ^{13}C NMR (CD_3OD, 125 MHz): δ_C (ppm) 155.9 (C-1), 96.4 (C-2/C-6), 154.8 (C-3/C-5), 134.6 (C-4), 56.7 (3,5-O$\underline{C}H_3$), 61.2 (4-O$\underline{C}H_3$), **glc**: 103.2 (C-1'), 74.9 (C-2'), 77.9 (C-3'), 71.6 (C-4'), 77.0 (C-5'), 68.7 (C-6'), **api**: 110.8 (C-1"), 77.8 (C-2"), 80.5 (C-3"), 74.9 (C-4"), and 65.4 (C-5"); ESI-MS m/z 477 $[M - H]^-$.

2.4. Cell lines and cell culture

The human hepatocyte carcinoma (HepG2), human prostate cancer (DU145), human lung cancer (A549), and human breast cancer (MCF7) cell lines were kindly provided by Prof. Jeong Hyung Lee, Department of Biochemistry, College of Natural Sciences, Kangwon National University, Republic of Korea. The cells were cultured at 37°C in an RMPI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator.

2.5. Cytotoxicity assay

Cytotoxicity of the samples was performed against four cancer cell lines using a previously reported procedure. The cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. The MTT assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity because the total mitochondrial activity is related to the number of viable cells. Briefly, the cells were seeded in 96-well plates at a concentration of 1×10^5 cells/well. After 24h, cells were treated with various concentrations of compounds (10, 30 and 100 μM), and incubated in a humidified 5% CO_2 atmosphere at 37°C. After 48h of incubation, 20 μL (0.5 mg/mL) of MTT was added to each well and incubated for another 4h. After removing the supernatant, formazan crystals were dissolved in isopropanol or DMSO, and the OD values were measured at 570 nm using a microplate reader. The IC_{50} values of the test samples in serial dilutions were calculated using nonlinear regression analysis (Table Curve2Dv4; AISN Software, Inc., Mapleton, OR, USA). Measurements were performed in triplicate and are representative of two independent experiments in which the values generally agreed within 10%. Camptothecin (0.1, 0.5, 1.0, and 5.0 μg/mL) was used as a positive control.

% Cell survival (%CS) is calculated as the following equation:

$$CS\% = \left[\frac{A_s - A_b}{A_c - A_b} \times 100\right]$$

Where:

As: Absorbance value of test compound;

Ab: Absorbance value of blank;

Ac: Absorbance value of control;

The test samples which have CS% value less than 50% are considered to have cytotoxicity and are selected to calculate IC_{50}.

% Cell inhibition = 100 - CS%

3. Results and discussion

Compound **1** was obtained as a white, amorphous powder, with a negative optical rotation $[\alpha]_D^{24}$ -122.3 (c 0.15, MeOH). Its molecular formula was found to be $C_{24}H_{38}O_{12}$

(six indices of hydrogen deficiency) as inferred from the HR-ESI-MS ion peak at *m/z* 541.2270 $[M + Na]^+$ and the ^{13}C NMR data. The ^1H and ^{13}C NMR spectrum of **1** revealed 24 carbon signals, 13 of which were assigned to a megastigmane skeleton, including four tertiary methyls [δ_H 2.21 (3H, s, H-10)/δ_C 26.7 (C-10), 1.18 (3H, s, H-11)/δ_C 32.6 (C-11), 1.39 (3H, s, H-12)/δ_C 30.1 (C-12), and 1.49 (3H, s, H-13)/δ_C 26.3 (C-13)], two sp^3 none-protonated carbons [δ_C 37.0 (C-1) and 78.5 (C-5)], one oxymethine [δ_H 4.30 (1H, tt, J = 11.5, 3.5 Hz, H-3)/δ_C 63.8 (C-3)], two methylenes [δ_H 1.92 (1H, ddd, J = 12.5, 3.5, 1.5 Hz, H-2a)/1.33 (1H, dd, J = 12.5, 11.0 Hz, H-2b)/δ_C 49.8 (C-2) and 2.48 (1H, ddd, J = 13.5, 5.5, 1.5 Hz, H-4a)/1.38 (1H, dd, J = 13.5, 11.5 Hz, H-4b)/δ_C 48.4 (C-4)], an α,β-unsaturated carbonyl [δ_C 200.9 (C-9)], and an allenic unit [δ_H 5.96 (1H, s, H-8)/101.3 (C-8), 213.1 (C-7), 118.7 (C-6)]. Additionally, signals from the disaccharide moieties were also observed (Table 1). Two anomeric proton signals [δ_H 4.52 (1H, d, J = 7.5 Hz, H-1') and 4.94 (1H, d, J = 2.5 Hz, H-1")] in the ^1H NMR spectrum suggested that these hexose and pentose moieties were in the pyranose and furanose forms, respectively. The sequential 1,2-*trans*-diaxial coupling patterns of H-1', H-2', H-3', H-4', and H-5' confirmed the hexose moiety to be β-glycopyranoside. A small coupling constant ($J_{1"\text{-}2"}$ = 2.5 Hz) confirmed the structure of the pentose unit to be β-apiofuranosyl. The deglycosylation position of **1** was reasonably assigned to C-5 rather than C-3 based on the downfield shift of one tertiary oxygenated carbon [δ_C 78.5 (C-5)]. Furthermore, attachment of the first sugar moiety at C-5 was also determined by an HMBC correlation of δ_H 4.52 (H-1') with δ_C 78.5 (C-5). The HMBC cross-peaks of another anomeric proton δ_H 4.94 (H-1") with δ_C 68.9 (C-6') of glucose indicated the connection of second sugar moiety to its megastigmane glucopyranoside framework via C-1"→C-6' (Fig. 2). This information was clearly expressed the downfield shift of oxygenated methylene carbon C-6' (δ_C 68.9) in glucopyranoside part. Based on the above evidence and comparison with reported literature [8], compound **1** was finally concluded to be pteleifoside C. This compound was previously obtained from the roots and rhizomes of *Melicope pteleifolia* (family Rutaceae) [8]. No activity was found in an inhibition assay for compound **1**.

Compound **2** was obtained as a pale yellow, amorphous solid. It has the molecular formula $C_{20}H_{18}O_8$ determined by the ESI-MS ion peak at *m/z* 409 $[M + Na]^+$ and the ^{13}C NMR data. The ^1H NMR spectroscopic data of **2** exhibited the characteristic peaks for a flavone skeleton. The signal at δ_H 6.73 (1H, s, H-8) was indicative of a proton on the A-ring, while the signals at δ_H 7.59 (1H, d, J = 1.8 Hz, H-2'), 6.93 (1H, d, J = 8.5 Hz, H-5'), and 7.66 (1H, dd, J = 8.5, 1.8 Hz, H-6') revealed three unsubstituted positions of an ABX aromatic ring system, corresponding to 3',4'-substituted flavonoid B-ring.

Table 1. ^1H and ^{13}C NMR spectroscopic data for **1** and reference compound

Pos.	$^{\#}\delta_C$ [a]	**1**	
		δ_C [a,b]	δ_H [a,c] mult. (*J* in Hz)
1	36.8	37.0	-
2	49.7	49.8	1.92 ddd (12.5, 3.5, 1.5) 1.33 dd (12.5, 11.0)
3	63.6	63.8	4.30 tt (11.5, 3.5)
4	48.3	48.4	2.48 ddd (13.5, 5.5, 1.5) 1.38 dd (13.5, 11.5)
5	78.3	78.5	-
6	118.5	118.7	-
7	212.9	213.1	-
8	101.2	101.3	5.96 s
9	200.7	200.9	-
10	26.5	26.7	2.21 s
11	32.8	32.6	1.18 s
12	30.0	30.1	1.39 s
13	26.2	26.3	1.49 s
1'	98.5	98.7	4.52 d (7.5)
2'	75.0	75.2	3.15 dd (9.0, 7.5)
3'	78.2	78.4	3.33 br d (9.0)
4'	71.3	71.6	3.24 t (9.0)
5'	76.4	76.6	3.32 m
6'	68.7	68.9	3.94 dd (11.5, 2.5) 3.52 dd (11.5, 5.5)
1"	110.8	111.0	4.94 d (2.5)
2"	77.9	78.1	3.90 d (2.5)
3"	79.8	80.5	-
4"	74.8	75.0	3.97 d (10.0) 3.78 d (10.0)
5"	65.5	65.7	3.59 br s

[a]Measured in CD_3OD, [b]125 MHz, [c]500 MHz, and $^{\#}\delta_C$ of pteleifoside C [8].

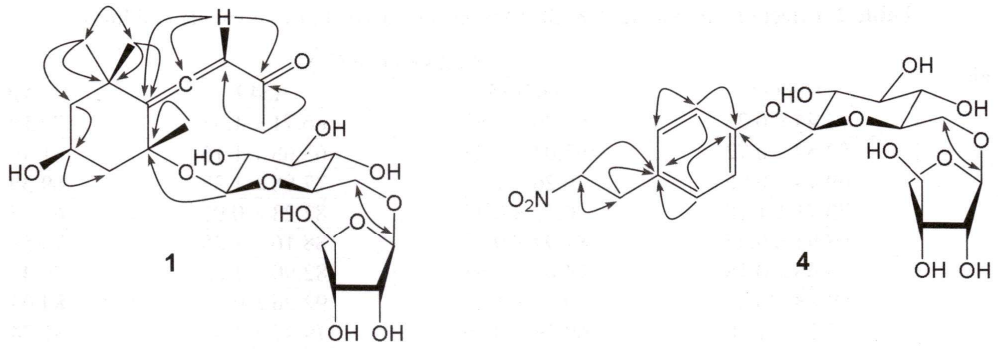

Fig. 2. Key HMBC correlations of **1** and **4**.

Also observed resonances for four methoxy substituents [δ_H 3.87 (3H, s, 3-OC$\underline{H}_3$), 3.96 (3H, s, 5-OC$\underline{H}_3$), 3.91 (3H, s, 6-OC$\underline{H}_3$), and 4.01 (3H, s, 7-OC$\underline{H}_3$)] and a two-proton singlet [δ_H 6.06 (2H, s, OC$\underline{H}_2$O)] characteristic of a methylenedioxy moiety in the ^{1}H NMR spectrum. The ^{13}C NMR and HSQC data showed 20 carbon signals as a flavanone carbon skeleton for this molecule, with characteristic C-ring resonances [δ_C 173.6 (C-4), 152.9 (C-2), and 140.8 (C-3)]. The presence of four methoxy groups [δ_C 59.9 (3-O$\underline{C}H_3$), 62.2 (5-O$\underline{C}H_3$), 61.5 (6-O$\underline{C}H_3$), and 56.3 (7-O$\underline{C}H_3$)], while the methylene signal [δ_C 101.6 (O$\underline{C}H_2$O)] confirmed that one aromatic ring was substituted with the methylenedioxy group. Besides, the 12 aromatic carbon signals that consisted of six oxygenated carbons [δ_C 152.4 (C-5), 140.2 (C-6), 157.7 (C-7), 153.5 (C-9), 147.9 (C-3'), and 149.4 (C-4')] and two nonprotonated carbons [δ_C 113.1 (C-10) and 124.6 (C-1')] indicated the presence of one trisubstituted aromatic ring. In the HMBC correlations of the singlet proton at δ_H 6.73 (H-8) with δ_C 140.2 (C-6), 157.7 (C-7), 153.5 (C-9), and 113.1 (C-10) allowed its placement at C-8 consistent with a pensubstituted A-ring. While the NOESY correlation of δ_H 6.73 (H-8) with δ_H 4.01 (7-OC$\underline{H}_3$) and the HMBC correlations from the δ_H 3.96 (5-OC$\underline{H}_3$) to δ_C 152.4 (C-5), δ_H 3.91 (6-OC$\underline{H}_3$) to δ_C 140.2 (C-6), which allowed assignment of three methoxy substituents at C-5, C-6, and C-7 of the A-ring. Further evidence for the B-ring substitution pattern of compound **2** was obtained by the HMBC correlation of H-2' with C-1', C-3', C-4', and H-6' with C-1', C-4', and C-5', from the dioxymethylene protons δ_H 6.06 to the two sp^2 carbons C-3' and C-4' permitted that a methylenedioxy substituent is attached at C-3'/C-4' of B-ring. The position of the remaining methoxy group was confirmed to attach at C-3 of the C-ring by the HMBC correlation from δ_H 3.87 to δ_C 140.8. From the above evidence and ESI-MS data, compound **2** was identified as melisimplexin (also called 3,5,6,7-tetramethoxy-3',4'-methylenedioxyflavone) [9]. Although compound **2** was also reported from *Melicope* species, it had too many origins [9]. Thus far, no bioassay has been reported from this compound.

Compound **4** was isolated as a white, amorphous powder. Its molecular formula was deduced to be $C_{19}H_{27}NO_{12}$ by HR-ESI-MS ion peaks at *m/z* 484.1440 $[M + Na]^+$, 496.1221 $[M + Cl]^-$ and ^{13}C NMR data, requiring the presence of seven degrees of unsaturation. The ^{1}H NMR spectroscopic data displayed signals for one set of protons from one 1,4-disubstituted aromatic ring [δ_H 7.20 (br d, J = 8.5 Hz, H-2/H-6) and 7.07 (br d, J = 8.5 Hz, H-3/H-5), with each one integrating for two protons], one methylene [δ_H 3.25 (2H, t, J = 7.0 Hz, H-7)], one oxymethylene [δ_H 4.69 (2H, t, J = 7.0 Hz, H-8)], together with two glycosidic moieties as evidenced by the presence of two anomeric proton signals [δ_H 4.85 (1H, d, J = 7.5 Hz, H-1') and 4.99 (1H, d, J = 2.5 Hz, H-1")], and other proton signals. Accordingly, ^{1}H NMR resonances of the sugar moieties of **4** almost exactly matched those of **1**, while the two compounds differed for the aglycon moiety. The ^{13}C NMR spectroscopic data of **4** gave a total of 19 carbon resonances, combined with the HSQC spectrum showed the presence of six aromatic carbons (four methines, one none-protonated carbon, and one oxygenated carbon), a set of two carbon signals of sp^3 methylene [δ_C 33.5 (C-7) and 77.5 (C-8)] was

Table 2. Effect of compounds **1-8** (30 μM) on cell survival of four cancer cell lines

Compounds	Cell survival (%)			
	HepG2	DU145	A549	MCF7
1	73.44 ± 0.25	67.90 ± 0.93	86.71 ± 1.33	79.19 ± 1.17
2	52.57 ± 0.72	65.03 ± 1.23	63.68 ± 1.16	61.46 ± 1.03
3	69.14 ± 0.96	76.26 ± 1.14	77.98 ± 1.37	69.39 ± 0.85
4	70.71 ± 1.18	70.18 ± 0.85	82.23 ± 0.91	66.35 ± 0.81
5	68.95 ± 0.47	81.94 ± 0.77	88.16 ± 1.35	76.58 ± 1.19
6	75.66 ± 0.15	74.05 ± 1.63	82.90 ± 1.03	70.47 ± 0.91
7	69.18 ± 0.43	70.15 ± 1.23	92.26 ± 0.88	84.03 ± 0.78
8	72.72 ± 1.28	69.18 ± 0.90	79.47 ± 1.92	87.74 ± 1.87
Camptothecin*	41.97 ± 0.42	22.89 ± 0.87	32.25 ± 0.83	34.89 ± 0.95

*Camptothecin (1.0 μg/mL) was used as a positive control.

assignable to an ethyl moiety. The A_2B_2 system, readily recognizable from its symmetry, helped identify the *p*-disubstituted benzene ring of the propenyl moiety in **4**. The remaining 11-carbon signals (seven methines, three methylenes, and one none-protonated carbon) were clearly identified that corresponded to the disaccharide core formed by β-glucopyranosyl and β-apiofuranosyl units with an api(1→6)glc linkage, as confirmed by the observed HMBC correlation H-1" of apinose with C-6' of glucose in **4** (Fig. 2). A detailed investigation of 2D NMR spectra supported the identification of **4** and comparison with NMR spectroscopic data in the published literature [10], compound **4** was established 6'-*O*-β-D-apiofuranosylthalictoside. Previous work on *Schisandra propinqua* var. *intermidia* by Wu T. identified the structure of **4** [10]. However, no studies regarding the biological activities of this compound are reported to date.

Other compounds were determined to be 3,5,6,7,3',4'-hexamethoxyflavone (**3**) [11], osmanthuside H (**5**) [12], icariside D_1 (**6**) [13], 3,4-dimethoxyphenol 1-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**7**) [14], and 3,4,5-trimethoxyphenol 1-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**8**) [15], by their good consistency with those reported in the literature. Previously, 3,5,6,7,3',4'-hexamethoxyflavone (**3**), also called 3-methoxysinensetin, was isolated from *Citrus sinensis* and found to display moderate to weak growth inhibitory effects against the KB, LNCaP, and LU1 cell lines [11]. Interestingly, in a side-by-side comparison of **3**, compound **2** had more origins and was distributed more wildly, which indicated compound **3** had more important taxonomic significance to *M. sinensis* than compound **2**. Similarly, in an investigation of *Osmanthus asiaticus* leaves, Sugiyama M. et. al reported the occurrence of **5** [12]. Meanwhile, another phenylethyl glycoside, icariside D_1 (**6**) isolated from *Epimedium grandiflorum* var. *thunbergianum* [13] and *Rosa damascena* [16]. In an initial study, compound **7** was originally isolated by Warashina T. and associates from the bark of *Tabebuia impetiginosa* [14], whereas metabolite **8** was found in the leaves and branches of *Morinda coreia* [15]. Unfortunately, although compounds **5-8** have been reported in many other plants, there are worth noting that still little research on their biological assay.

The cytotoxicity of the isolated compounds **1-8** was evaluated by screening their potencies to inhibit HepG2, DU145, A549, and MCF7 cancer cell lines, using an MTT assay. These results demonstrate that compounds **1-8** exhibited weak cytotoxic activity on four cell lines. In the presence of compounds **1-8**, cell survival percentages were observed ranging from 52.57 ± 0.72% to 75.66 ± 0.15% (HepG2), 65.03 ± 1.23% to 81.94 ± 0.77% (DU145), 63.68 ± 1.16% to 88.16 ± 1.35% (A549), 61.46 ± 1.03% to 87.74 ± 1.87% (MCF7) cell lines, using camptothecin as a positive control (Table 2).

4. Conclusions

The phytoconstituents of *M. sinensis* leaves have been demonstrated in the present study, eight compounds were isolated, including pteleifoside C (**1**), melisimplexin (**2**), 3,5,6,7,3',4'-hexamethoxyflavone (**3**), 6'-*O*-β-D-apiofuranosylthalictoside (**4**), osmanthuside H (**5**), icariside D_1 (**6**), 3,4-dimethoxyphenol 1-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**7**), and 3,4,5-trimethoxyphenol 1-*O*-β-D-apiofuranosyl-

(1→6)-β-D-glucopyranoside (**8**). The structures of these compounds were elucidated by NMR spectroscopic analysis and comparison with the reported data. Among these, phenolic glycosides are the predominant compounds. Compounds **1-8** showed weak or fewer effects against the tested cancer cell lines. This is the first time that glycoside constituents and their cytotoxic activity were reported from *M. sinensis*, which was established based on the extensive literature search in the SciFinder database. Further studies should focus on the diversity of metabolites of the genus *Miliusa*.

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CYCLOPEPTIDES FROM THE MARINE ACTINOMYCETE *DIETZIA* SP. G727 DERIVED FROM THE SEAWEED *LOBOPHORA* SP.

Nguyen Thi Hue[1], Do Thi Quynh[1], Nguyen Mai Anh[1], Le Thi Hong Minh[1], Phi Thi Dao[1], Nguyen Thuy Linh[1], Doan Thi Mai Huong[1,2,*], Pham Van Cuong[1,2,*]

[1]Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST);

[2]Faculty of Chemistry, Graduate University of Science and Technology, VAST

*Corresponding author: huongdm@imbc.vast.vn or phamvc@imbc.vast.vn

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Summary

Cyclopeptides from the Marine Actinomycete *Dietzia* sp. G727 Derived from the Seaweed *Lobophora* sp.

From the methanol extract of the *Dietzia* sp. G727 derived from the seaweed *Lobophora* sp., seven compounds were isolated including staphylopeptide A (**1**), cyclo-(L-Pro-L-Asn) (**2**), cyclo-(L-Pro-L-Ser) (**3**), cyclo-(L-Pro-L-Thr) (**4**), cyclo-(L-Ala-4-hydroxy-L-Pro) (**5**), adenine (**6**) and adenosine (**7**). Their structures were elucidated by the nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS) and comparison with the reference data. Compounds **3**, **5** and **6** showed good antimicrobial activity against *Enterococcus faecalis* and *Candida albicans* with the MIC values in the range from 32 to 128 µg/mL. In addition, only compound **6** inhibited Gram-negative bacteria *Escherichia coli* with a MIC value of 64 µg/mL. To the best of our knowledge, this is the first report of the isolation of staphylopeptide A from *Dietzia* genus.

Keywords: *Dietzia genus, Marine actinomycete, Cyclodipeptide, Cyclotetrapeptide, Antimicrobial activity.*