

Flavonoids from *Cleistocalyx operculatus* Buds and their Cytotoxic Activity

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Cleistocalyx operculatus (Roxb.) Merr and Perry (Myrtaceae) is a well-known medicinal plant, widely distributed and propagated in China, Vietnam and some other tropical countries. Several species of *Cleistocalyx* are used in folk medicine. In Vietnam, *C. operculatus* is commonly called "Voi". The flower buds ("Nu Voi") and leaves ("La Voi") have been used to make a beverage since ancient times.¹ The buds are commonly used as an ingredient for tonic drinks in Southern China.² The water extract of *C. operculatus* buds was shown to increase the contractility and decrease the frequency of contraction in an isolated rat heart perfusion system.³ It showed strong protective effects on lipid peroxidation in rat liver microsomes and on the H₂O₂-induced trauma of PC12 cells.⁴ *C. operculatus* extract showed inhibitory activity against the α -glucosidase, rat-intestinal maltase, sucrase activities, and is considered a promising material for preventing and treating diabetes.⁵ Recently, the essential oil of the *C. operculatus* buds was investigated for its *in vitro* and *in vivo* anti-inflammatory activities. These results suggested that its essential oil might exert an anti-inflammatory effect by suppressing the expression of pro-inflammatory cytokines, which is mediated, at least in part, by blocking the NF- κ B activation.⁶ Previous phytochemical attention has led to the characterization of oleanane-type triterpenes,^{7,8} and flavonoids.² Analysis of its leaf oil by gas chromatography (GC) and GC-mass spectroscopy (GC/MS) has also been reported.⁹ Chalcone compounds from this plant possessed antioxidant and anticancer activities.¹⁰⁻¹² In our previous study, 3'-formyl-4,4',6'-trihydroxy-2'-methoxy-5'-methylchalcone, 3'-formyl-4,6'-dihydroxy-2'-methoxy-5'-methylchalcone 4'-O- β -D-glucopyranoside, (2*S*)-8-formyl-6-methylnaringenin, and (2*S*)-8-formyl-6-methylnaringenin 7-O- β -D-glucopyranoside were isolated from EtOAc-soluble fraction and their radical scavenging activities were reported.¹³ In the present study, further phytochemical investigation of the water-soluble fraction of this plant led to the isolation of a new flavonoid glycosides (**1**), and six known compounds (**2-7**). Details of the isolation, structural determination and cytotoxic activity are described herein.

The MeOH extract of the buds of *C. operculatus* was partitioned into hexane-, EtOAc-, and water-soluble fractions. Chromatographic purification of the water-soluble fraction led to the isolation of seven compounds (**1-7**) (Fig. 1). Six of these were identified as gossypetin-8,3'-dimethylether-3-O- β -D-galactoside (**2**), myricetin-3'-methylether-3-O- β -D-galactopyranoside (**3**), myricetin-3'-methylether (**4**), quercetin (**5**), kaempferol (**6**), and tamarixetin (**7**) by comparing their physical and spectroscopic data with previous reported papers.¹⁴⁻¹⁷

Compound **1** was isolated as a yellow amorphous powder and supported a positive ferric chloride reaction. The positive HR-FAB-MS analysis of **1** indicated an ion peak $[M + Na]^+$ at m/z 547.1068, which corresponded to the molecular formula C₂₃H₂₄O₁₄. The IR absorptions at 3320 and 1660 cm⁻¹ showed the presence of hydroxy and carbonyl groups, respectively, and the UV spectrum displayed two maximum bands at 262 and 381 nm, characteristic of a flavon-3-ol.^{16,17} The ¹H NMR spectrum of **1** indicated the presence of two methoxy groups at δ 3.97 (6H, s), proton signals were also observed at δ 6.90 (1H, s, H-6) and 8.17 (2H, s, H-2', 6'). In addition, the ¹H NMR spectrum showed seven characteristic signals typical of a sugar moiety, including a peak at δ 6.41 (1H, d, $J = 7.6$ Hz) for an anomeric proton (H-1''). The ¹³C NMR and DEPT spectra of **1** showed 23 signals, including nine oxygenated carbons, one carbonyl carbon at δ 179.6 (C=O), two methoxy carbons, and six carbon signals between δ 62.4 and 104.6, which could be assigned to a sugar unit. The full NMR assignments and connectivities of **1** were determined by HMQC and HMBC spectroscopic data analysis. The HMBC spectrum confirmed the correlations between methoxy protons at δ 3.97 (6H, s) and C-3' (δ 149.3), and at δ 3.97 (6H, s) and C-5' (δ 149.2). Furthermore, correlations also observed in the NOESY spectrum of **1** (Fig. 2), indicated that the two methoxy groups in **1** were placed at the C-3' and

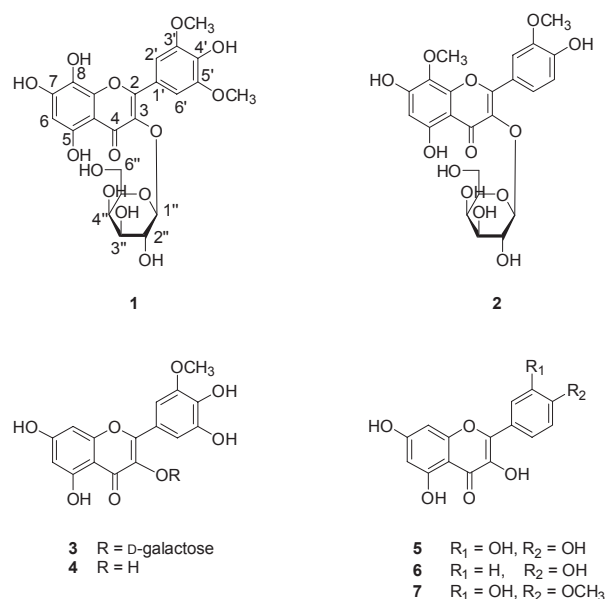


Figure 1. Chemical structures of isolated compounds **1-7**.

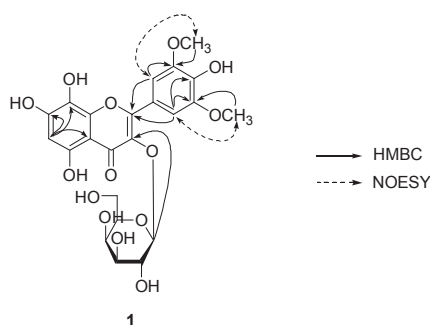


Figure 2. Selected HMBC and NOESY correlations of compound **1**.

Table 1. Cytotoxic activity of isolated compounds against cancer cell lines

Compounds	IC ₅₀ (μM)		
	Hela	HL-60	A549
1	38.7	16.5	> 100
2	42.1	18.1	> 100
3	45.5	15.7	> 100
Adriamycin ^a	0.70	0.18	5.72

^aUsed as positive control.

C-5' positions. The correlation from the proton signal at δ 6.90 (1H, s) to the three quaternary carbons at δ 105.7 (C-4a), 127.5 (C-8) and 155.7 (C-7) placed the location of this aromatic proton at C-6 (Fig. 2). The hydroxyl group was placed at C-8 as this carbon signal was shifted upfield (127.5 ppm) in its ¹³C-NMR spectrum.¹⁶ In addition, the two aromatic protons at positions C-2' and C-6' were further supported by the HMBC correlations between δ 8.17 (2H, s) and δ 157.9 (C-2). To identify the sugar moiety, acid hydrolysis of **1** yielded D-galactose, as confirmed by co-TLC with an authentic sample and in combination with NMR data interpretation. The $J_{H,H}$ value (7.6 Hz) of the anomeric proton (H-1'') indicated that galactose was linked *via* a β -linkage.¹⁶ In addition, the aromatic signal was shifted downfield at δ 135.5 (C-3), which supported the location of galactose at this position. This finding was confirmed by the HMBC correlation between H-1'' and C-3 (Fig. 2). Following the unambiguous NMR data assignment, the sugar moiety was further established by HMQC experiments. Thus, the structure of **1** was established as 5,7,8,4'-tetrahydroxy-3',5'-dimethoxyflavone-3-O- β -D-galactopyranoside.

Compounds **1-3** with a galactose moiety in the structures were evaluated for their *in vitro* cytotoxic activity against Hela, HL-60, and A549 cancer cell lines using MTT assay method with slight modification.¹⁸ As shown in the results presented in Table 1, compounds **1-3** showed weak cytotoxic activity against Hela cancer cell lines with IC₅₀ values of 38.7, 42.1 and 45.5 μ M, respectively. In the case of HL-60, these isolates displayed moderate cytotoxic activity with IC₅₀ values ranging from of 13.7 to 18.1 μ M. However, they showed very weak inhibitory activity against A549 cell lines with IC₅₀ values over than 100 μ M. Plochmann *et al.* investigated the effects of methoxylation on cytotoxic activity by comparing the toxic affects of these

compounds with unmethoxylated or less-methoxylated molecules. The higher methoxylated compounds were significantly more toxic than the less methoxylated molecules.¹⁹ Our results revealed that the cytotoxic activity of flavones decreased when 3'-hydroxyl was methylated (**1-3**), while the effect of 7-OH on the cytotoxic activity was uncertain. Furthermore, the existence of 3-Gal seemed to attenuate the cytotoxic activity of these compounds, suggesting that the sugar moiety could reduce the cytotoxic activity and that glycoside had a weaker cytotoxic activity than the corresponding aglycones, respectively.

Experimental

General experimental procedures. UV spectra were recorded on a JASCO V-530 spectrophotometer. IR spectra were obtained on a JASCO FT/IR 300-E spectrometer. NMR experiments were conducted on a Varian Unity INOVA 400 spectrometer. ¹H and ¹³C NMR spectra were recorded at 400 and 100 MHz, respectively, and tetramethylsilane was used as the internal standard. FAB-MS and HR-FAB-MS analyses were performed on a Micromass QTQF2 mass spectrometer. TLC was carried out on Merck silica gel F₂₅₄-precoated glass plates and RP-18 F_{254S} plates. HPLC was performed on a Waters 600E multi-solvent delivery system connected to a UV detector using RS Tech Optima Pak C18 column (10 \times 250 mm, 10 μ m particle size) semi-preparative columns.

Plant material. The buds of *C. operculatus* were purchased in Dong Xuan herbarium market, Hanoi, Vietnam, in July 2007 and identified by Professor Pham Thanh Ky, Department of Pharmacognosy, Hanoi University of Pharmacy. A voucher specimen (0160) was deposited in the herbarium of the Hanoi University of Pharmacy.

Extraction and isolation. The buds (1.8 kg) were extracted three times (3 h \times 3 L) with refluxing methanol. The MeOH extract was combined and concentrated to yield a residue which was suspended in water and then successively partitioned with *n*-hexane, EtOAc, and water residue. The water layer (18.0 g) was separated by Sephadex LH-20 silica gel column chromatography using a gradient of MeOH-H₂O (from 40:60 to 100:0), to yield seventeen subfractions (W1 ~ W17) according to their TLC profiles. Sub-fraction W5 (0.5 g) was purified by semi-preparative HPLC systems [mobile phase (10 to 75% MeOH in water for 60 min), flow rate 5 mL/min; UV-detection at 254 nm] resulted in the isolation of compounds **5** (12.5 mg; t_R = 38.5 min), **6** (10.0 mg; t_R = 41.3 min), and **7** (11.0 mg; t_R = 45.0 min). The sub-fraction W11 (0.25 g) was further purified by semi preparative HPLC [mobile phase (10 to 75% MeOH in water + 0.1% Trifluoroacetic acid for 60 min), flow rate 5 mL/min; UV-detection at 254 nm] resulted in the isolation of compound **2** (2.8 mg; t_R = 37.4 min). The sub-fraction W14 (0.68 g) was further purified by semi preparative HPLC [mobile phase (15 to 65% MeOH in water + 0.1% Trifluoroacetic acid for 60 min), flow rate 5 mL/min; UV-detection at 254 nm] obtained compounds **1** (19.8 mg; t_R = 32.4 min), **3** (6.0 mg; t_R = 40.1 min), and **4** (5.4mg; t_R = 43.2 min).

5,7,8,4'-Tetrahydroxy-3',5'-dimethoxyflavone-3-O- β -D-galactopyranoside (1**):** Yellow amorphous powder; UV λ_{max} (MeOH): 262, 381 nm; IR (KBr) cm⁻¹ 3320, 2950, 2369, 1660;

FAB-MS m/z 547.1 $[M + Na]^+$; HR-FAB-MS m/z 547.1068 $[M + Na]^+$ (calcd for $C_{23}H_{24}O_{14}Na$, 547.1064). 1H NMR (400 MHz, C_5D_5N) δ 4.19–4.77 (1H, t, $J = 8.0$ Hz, H-2''); 1H, dd, $J = 2.8, 8.0$ Hz, H-3''; 1H, d, $J = 2.8$ Hz, H-4''; 1H, t, $J = 6.0$ Hz, H-5''; 1H, dd, $J = 6.0, 10.8$ Hz, H-6''a; 1H, dd, $J = 6.4, 10.8$ Hz, H-6''b), 3.97 (6H, s, OCH_3), 6.41 (1H, d, $J = 7.6$ Hz, H-1''), 6.90 (1H, d, $J = 2.0$ Hz, H-6), 8.17 (2H, s, H-2',6'). ^{13}C NMR (100 MHz, C_5D_5N) δ 57.3 (3',5'- OCH_3), 62.4 (C-6''), 70.3 (C-4''), 73.9 (C-2''), 75.3 (C-3''), 78.1 (C-5''), 100.4 (C-6), 104.6 (C-1''), 105.7 (C-4a), 108.8 (C-2'), 108.8 (C-6'), 121.5 (C-1'), 127.5 (C-8), 135.5 (C-3), 141.2 (C-4'), 149.2 (C-5'), 149.3 (C-3'), 146.8 (C-8a), 155.2 (C-5), 155.7 (C-7), 157.9 (C-2), 179.6 (C-4).

Determination of sugar component. The monosaccharide subunit of **1** was obtained by acid hydrolysis. Compound **1** (4 mg) in 10% HCl-dioxane (1:1, 1 mL) was heated at 80 °C for 4 h in a water bath. The reaction mixtures were neutralized with Ag_2CO_3 , filtered, and then extracted with EtOAc. After concentration, each H_2O layer (monosaccharide portion) was evaporated in vacuo to give residue, which was subjected to a silica gel column chromatography ($CHCl_3$ -MeOH- H_2O (55:45:10) to yield D-galactose. The sugar was compared with authentic sample by TLC. The R_f value for the above sugar was 0.19.

Cytotoxic activity assay. The cancer cell lines were maintained in RPMI 1640, which included l-glutamine with 10% FBS and 2% penicillin-streptomycin. Cells were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxic activity was measured using a modified MTT assay.¹⁸ Viable cells were seeded in the growth medium (100 μ L) into 96-well microtiter plates (1×10^4 cells per well) and incubated at 37 °C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5.0 to 150 μ M by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing for 24 h, 10 μ L of the test sample was added to each well. The same volume of DMSO was added to the control wells. On removing medium after 48 h of the test sample treatment, MTT (5 mg/mL, 10 μ L) was also added to the each well. After 4 h incubation, the plates were removed, and the resulting formazan crystals were dissolved in DMSO (150 μ L). The OD

was measured at 570 nm. The IC_{50} value was defined as the concentration of sample that reduced absorbance by 50% relative to the vehicle-treated control.

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