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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 H_2O_2-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-κB 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 $C_{23}H_{24}O_{14}$. 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 ^{1}H 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 ^{1}H 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 ^{13}C 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

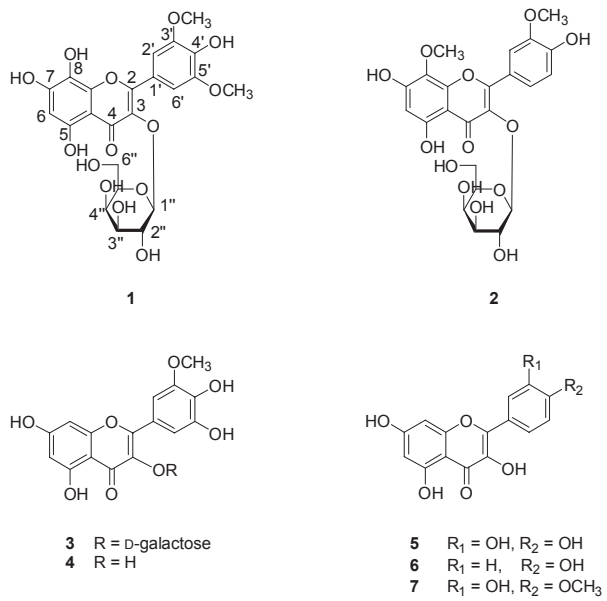

Figure 1. Chemical structures of isolated compounds **1-7**.

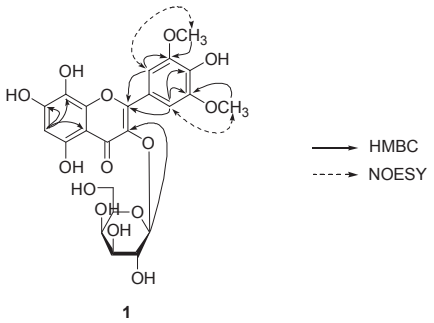

Figure 2. Selected HMBC and NOESY correlations of compound **1**.

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

[a]Used 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 ^{13}C-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 IC_{50} values of 38.7, 42.1 and 45.5 μM, respectively. In the case of HL-60, these isolates displayed moderate cytotoxic activity with IC_{50} values ranging from of 13.7 to 18.1 μM. However, they showed very weak inhibitory activity against A549 cell lines with IC_{50} values over than 100 μM. 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. ^{1}H and ^{13}C 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 F_{254}-precoated glass plates and RP-18 F_{254S} plates. HPLC was performed on a Waters 600E multisolvent delivery system connected to a UV detector using RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm 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 × 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-H_2O (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 μL) 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 μM 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 μL 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 μL) 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 μL). 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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