Antioxidative Flavonoids from *Cleistocalyx operculatus* Buds

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Four new flavonoids, 3’-formyl-4’,6’,4-trihydroxy-2’-methoxy-5’-methylchalcone (1), 3’-formyl-6’,4-dihydroxy-2’-methoxy-5’-methylchalcone 4’-O-β-D-glucopyranoside (2), (2S)-8-formyl-6-methylnaringenin (3), and (2S)-8-formyl-6-methylnaringenin 7-O-β-D-glucopyranoside (4) were isolated from the buds of *Cleistocalyx operculatus* (Myrtaceae). The structures of the new metabolites (1—4) were determined on the basis of spectroscopic analyses including 2 dimensional NMR. Compounds 1 and 3 exhibited 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity with IC50 values of 22.8 and 27.1 μM, respectively.

Key words *Cleistocalyx operculatus*; Myrtaceae; anti oxidant; flavonoid

*Cleistocalyx operculatus* (Roxb.) Merr. et Perry (Myrtaceae) is a large green tree that grows in rural areas of North Vietnam where it is commonly called Vôi. The flower buds (name as Nu Vôi) and leaves (La Vôi) have been used to make a beverage since ancient times.1 It is also a well-known medicinal plant whose buds are commonly used as an ingredient for tonic drinks in Southern China.2 The water extract of the buds of *C. operculatus* has been 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 the H2O2-induced trauma of rat pheochromocytoma (PC12).4 It is also a well-known medicinal plant whose buds are commonly used as an ingredient for tonic drinks in Southern China.2 The water extract of the buds of *C. operculatus* has been 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 the H2O2-induced trauma of rat pheochromocytoma (PC12) cells.4 *C. operculatus* extract showed inhibitory activity against the α-glucosidase, rat-intestinal maltase, and sucrase activities. It is also considered a promising material for preventing and treating diabetes.5 Previous phytochemical attention characterized the oleanane-type triterpenes,6,7) and flavonoids.2,8) Analysis of its leaf oil by gas chromatography (GC) and chromatography/mass spectrometry (GC/MS) has confirmed the correlations between aldehyde proton (δ 10.41, s) and C-3’ (δ 108.7), between methoxy protons (δ 3.82, s) and C-2’ (δ 165.7), and between methyl protons (δ 2.04, s) and C-5’ (δ 110.2) (Fig. 2). Based on the above analyses, the structure of compound 1 was elucidated as 3’-formyl-4’,6’,4-trihydroxy-2’-methoxy-5’-methylchalcone.

**Results and Discussion**

The MeOH extract of the buds of *C. operculatus* was partitioned into hexane-, EtOAc-, and BuOH-soluble fractions. Chromatographic purification of the EtOAc-soluble fraction led to the isolation of four compounds (1—4) (Fig. 1).

Compound 1 was isolated as a yellow amorphous powder. In the positive ion mode, electrospray ionization mass spectrometry (ESI-MS), a quasimolecular ion peak at m/z 329 [M+H]+ was observed, and high-resolution ESI-MS (HR-ESI-MS) analysis indicated an ion peak at 329.1813 which corresponded to the molecular formula, C18H16O6. The infrared (IR) absorptions at 3452, 2873 and 2758 cm⁻¹ showed the presence of hydroxyl and aldehyde groups. The 1H nuclear magnetic resonance (NMR) spectrum of 1 indicated the presence of methoxy group at δ 3.82 (3H, s), aldehyde group at δ 10.41 (1H, s), and methyl group at δ 2.04 (3H, s). Proton signals were also observed at δ 7.54 (2H, dd, J=8.5, 2.0 Hz, H-2, 6) and δ 6.94 (2H, dd, J=8.5, 1.8 Hz, H-3, 5) and assigned to 4-substituted phenyl in the B ring of chalcone.9 An AB spin system with J=15.8 Hz at δ 7.73 and 7.78 indicated the presence of protons of an α,β-unsaturated ketone moiety.10) The 13C-NMR and distortionless enhancement by polarization transfer (DEPT) spectrum of 1 showed 18 signals, including three oxygenated aromatic carbons at δ 165.7 (C-2’), 169.8 (C-6’) and 160.7 (C-4), one carbonyl carbon at δ 139.8 (C=O), one aldehyde carbon at δ 191.7 (CHO), one methyl carbon at δ 10.6 (5’-CH3), and four aromatic carbons at δ 130.2 (C-2, 6) and 118.3 (C-3, 5). The 1H- and 13C-NMR spectra (Table 1) were very similar to those of 3’-formyl-4’,6’-dihydroxy-2’-methoxy-5’-methylchalcone,11) except for the hydroxy group at the position C-4 of the B ring. The full NMR assignments and connectivities of 1 were determined by heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC) spectroscopic data analyses. The HMBC spectrum confirmed the correlations between aldehyde proton (δ 10.41, s) and C-3’ (δ 108.7), between methoxy protons (δ 3.82, s) and C-2’ (δ 165.7), and between methyl protons (δ 2.04, s) and C-5’ (δ 110.2) (Fig. 2). Based on the above analyses, the structure of compound 1 was elucidated as 3’-formyl-4’,6’,4-trihydroxy-2’-methoxy-5’-methylchalcone.

![Chemical Structures of Isolated Compounds](image-url)
Compound 2 was isolated as a yellow amorphous powder with a specific rotation $[\alpha]_{D}^2$ of $-125.5$ (c=0.2, MeOH). The HR-ESI-MS of 2 showed an [M+H]$^+$ peak at $m/z$ 491.1585, which established a molecular formula of C$_{24}$H$_{26}$O$_{11}$, indicating that it was a glycosylated derivative of 1. Its $^1$H-NMR spectrum showed seven new characteristic signals typical of a sugar moiety, including a peak at $\delta$ 5.08 for an anomic proton (H-1$^\alpha$). The $^1$C-NMR spectrum also exhibited six carbon signals between $\delta$ 62.9 and 101.7, in addition to the signals assignable to 1. The sugar was assigned as glucopyranose on the basis of NMR data and the $R_f$ value compared with authentic glucose after enzymatic (naringinase) hydrolysis of 2. The absolute configuration was determined to be $\beta$-glucose by GC. The $J_{IH}$ value (7.8 Hz) of the anomic proton (H-1$^\alpha$) indicated that glucose was linked via a $\beta$-linkage. In addition, the aromatic signal was shifted downfield at $\delta$ 166.8 (C-4$^\gamma$), which suggested that the OH group at C-4$^\gamma$ of 1 was glucosylated in 2. This finding was confirmed by the HMBC correlation between H-1$^\alpha$ and C-4$^\gamma$ (Fig. 2). The NMR data were unambiguously assigned for both the aglycone and the sugar moiety by HMOC experiments. The structure of 2 was therefore established as 3'- formyl-6',4-dihydroxy-2'-methoxy-5'-methylchalcone 4'-O-$\beta$-d-glucopyranoside.

Compound 3 was obtained as a yellow amorphous powder. Its molecular formula was found to be C$_{17}$H$_{14}$O$_{6}$ by the HR-ESI-MS ($m/z$ 315.2788 [M+H]$^+$). The UV spectrum exhibited an absorption maxima at 270 and 335 nm, which suggested a flavanone nature for 3. Compound 3 displayed aromatic signals downfield at $\delta$ 7.50 (2H, dd, $J=8.5, 2.2$ Hz, H-2'), 6.88 (2H, dd, $J=8.5, 2.0$ Hz, H-3', 5'), $\delta$ 127.0 (C-2', 6'), 114.8 (C-3', 5') and 159.2 (C-4') in the $^1$H- and $^1$C-NMR spectra, suggesting the hydroxyenated position to be at C-4'. Remarkable differences were observed between the $^1$H- and $^1$C-NMR spectra of compound 3 (Table 2) and those of compounds 1 and 2 (Table 1). The three one-proton coupled double doublets at $\delta$ 5.41 (1H, dd, $J=12.5, 2.4$ Hz, H-2), $\delta$ 2.91 (1H, dd, $J=16.5, 12.5$ Hz, H-3ax), and $\delta$ 2.58 (1H, dd, $J=16.5, 2.4$ Hz, H-3eq) suggested that the C ring was saturated. This splitting pattern was due to the coupling between the H-2 axial proton and the H-3 geminal protons. The absolute configuration at the C-2 stereocenter was established to be S on the basis of the high-amplitude, negative Cotton effect in the 270—300 nm region and the weak positive Cotton effect in the 325—350 nm region, which indicated that stereospecificity was achieved in the process of C-ring cyclization. The structure of 3 was also established by
Table 3. DPPH Radical Scavenging Activity of Compounds 1–4

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC_{50} (μM)</th>
<th>α-Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>117.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>27.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>105.8</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td></td>
<td>20.1</td>
</tr>
</tbody>
</table>

a) IC_{50} values were calculated from regression lines using five different concentrations in triplicate. b) Positive control.

Comparing the 1D and 2D NMR spectra, including HMBC (Fig. 2), with those of (2S)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone isolated from the same plant, 2) Thus, the structure of 3 was determined as (2S)-8-formyl-6-methyl-naringenin.

Compound 4 was obtained as a white amorphous powder. The HR-ESI-MS spectrum showed the [M+H]^+ peak at m/z 477.2594, which established a molecular formula of C_{23}H_{24}O_{11}, indicating that it was a glycosylated derivative of 3. In addition to all the signals assignable to 3, the 1H- and 13C-NMR spectra of 4 showed seven and six characteristic signals of a sugar moiety in the region ranging from δ 3.27 to 4.96, and from δ 6.25 to 10.16, respectively. The sugar was assigned as glucopyranose on the basis of NMR data and the comparison of the Rf value with authentic glucose after enzymatic (naringinase) hydrolysis of 4. The absolute configuration was determined to be D-glucose by GC. The J_{HH} value (7.5 Hz) of the anomeric proton (H-1) indicated that glucose was linked via a β-linkage. The location of glucose was confirmed by HMBC correlation between H-1 and C-7 (Fig. 2).

On the basis of the data, the structure of 4 was assigned as (2S)-8-formyl-6-methyl-naringenin 7-O-β-D-glucopyranoside.

Compounds 1–4 were tested for their in vitro antioxidant activity using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Table 3). Compounds 1 and 3 exhibited DPPH radical scavenging activity with inhibitory concentration (IC_{50}) values of 22.8 and 27.1 μM, respectively, while compounds 2 and 4 exhibited weak activity with IC_{50} values of 117.2 and 105.8 μM, respectively.

Experimental

General Experimental Procedures Optical rotations were measured with a JASCO DIP 1000 digital polarimeter. UV spectra were recorded on a JASCO V-530 spectrophotometer, and CD spectra were recorded on a JASCO J-715 CD ORD spectropolarimeter. 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. ESI-MS and HR-ESI-MS analyses were performed on a Micromass QTQF2 mass spectrometer. EI-MS spectra were obtained at 70 eV, using BCl_3 as the internal standard. ESI-MS and HR-ESI-MS analyses were performed on a Micromass QTQF2 mass spectrometer. EI-MS spectra were obtained at 70 eV, using BCl_3 as the internal standard.

Extraction and Isolation The buds (0.8 kg) were extracted with 2 l of hexane–EtOAc (9:1). The extract was evaporated to dryness. The residue was chromatographed on a preparative silica gel column (170 mm, i.d.) eluting with hexane–EtOAc (9:1). The hexane fraction was evaporated to dryness and subjected to column chromatography on silica gel (200–300 mesh), eluting with benzene, EtOAc, and BuOH. The EtOAc-soluble fraction (15.3 g) was separated by silica gel column chromatography using a gradient of hexane–EtOAc (from 30:1 to 5:1), then EtOAc–MeOH (from 20:1 to 1:1), to yield five fractions (E1—E5) according to their TLC profiles. Fraction E4 (0.8 g) was chromatographed over silica gel column using a gradient of hexane–EtOAc–MeOH (from 10:1 to 5:1), to yield five subfractions E4.1—E4.5. The E4.3 fraction was further purified by semi preparative HPLC (Shimadzu Prominence) using a stepwise gradient of MeOH–H_2O (from 50:50 to 100:0 for each step), to afford ten subfractions (E5.1—E5.10). Fraction E5.3 was purified by semi preparative HPLC using the same condition above except that the gradient of mobile phase (50% to 100% MeOH in water over 50 min) to afford compound 2 (14.1 mg, t_R = 18.2 min). Fraction E5.5 was purified by semi preparative HPLC using the same condition with that employed for compound 2 to afford compound 4 (12.7 mg, t_R = 22.8 min).

3′-Formyl-4′,6′-dihydroxy-2′-methoxy-5′-methylethalcone (1): Yellow amorphous powder; UV λ_{max} (MeOH) nm (ε): 280 (4.12), 312 (3.87); IR (KBr) cm⁻¹: 3452, 2873, 2758, 1620, 1547, 1465, 780, 705; ESI-MS m/z: 329 [M+H]^+; HR-ESI-MS m/z: 329.1813 [M+H]^+ (Calcd for C_{18}H_{16}O_{6}: 329.1820); for 1H- and 13C-NMR spectral data, see Table 1.

4′-β,β′-D-Glucopyranosyl-4′-O-β,β′-Glucopyranosyl compound 2: Yellow amorphous powder; [α]_D = −125.5 (c=0.2, MeOH); UV λ_{max} (MeOH) nm (ε): 285 (4.05), 325 (3.80); IR (KBr) cm⁻¹: 3400, 2958, 2760, 1625, 1606, 1518, 1416, 1280, 1175, 1076 cm⁻¹; ESI-MS m/z: 491 [M+H]^+; HR-ESI-MS m/z: 491.1585 [M+H]^+ (Calcd for C_{23}H_{24}O_{11}: 491.1586); for 1H- and 13C-NMR spectral data, see Table 1.

(2S)-8-Formyl-6-methyl-naringenin (3): Yellow amorphous powder; [α]_D = +58.8 (c=0.2, MeOH); UV λ_{max} (MeOH) nm (ε): 232 (4.15), 270 (4.37), 385 (9.50); IR (KBr) cm⁻¹: 3425, 2827, 2770, 1686, 1630, 1527, 1385, 1278, 1090; ESI-MS m/z: 315 [M+H]^+; HR-ESI-MS m/z: 315.2788 [M+H]^+ (Calcd for C_{22}H_{20}O_{5}: 315.2790); for 1H- and 13C-NMR spectral data, see Table 2.

Enzymatic Hydrolysis of 2 and 4 Naringinase (100 mg, from Pseudomonas aeruginosa) was added to a suspension of 2 and 4 (5 mg) in 50 mM acetate buffer (pH 5.5), and the mixture was stirred at 37 °C for 5 h. The reaction mixture was extracted with EtOAc (10 ml×3), and the organic layer was evaporated to dryness. The residue was chromatographed on a preparative TLC with CHCl_3–MeOH (9:1) to give 1 (1.5 mg, t_R = 3430, 2872, 2770, 1685, 1640, 1529, 1380, 1276, 1086; ESI-MS m/z: 477 [M+H]^+), IR-ESI-MS m/z: 477.2590 [M+H]^+ (Calcd for C_{22}H_{20}O_{5}: 477.2594); for 1H- and 13C-NMR spectral data, see Table 2.

References