Two flavonoids first isolated from the seed of *Syzygium nervosum* and preliminary study of their anticancer and anti-HIV-1 reverse transcriptase activities

Anuruk Chailungka¹, Thiti Junpirom¹, Wilart Pompimon², Narong Nuntasaen³ and Puttinan Meepowpan¹, *

¹ Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
² Laboratory of Natural Products, Centre for Innovation in Chemistry, Faculty of Science, Lampang Rajabhat University, Lampang 52100, Thailand
³ The Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok 10900, Thailand
* Corresponding author, e-mail: pmeepowpan@gmail.com

Received: 5 November 2015 / Accepted: 30 March 2017 / Published: 5 April 2017

**Abstract:** Two flavonoids, viz. 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC) and hariganetin were first isolated from the dichloromethane extract of the seed of *Syzygium nervosum* and their biological activities against cancer cell lines and HIV-1 reverse transcriptase were preliminary studied. Both DMC and hariganetin show moderate activities against HIV-1 reverse transcriptase with 67.5% and 64.1% inhibition respectively.

**Keywords:** 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone, hariganetin, *Syzygium nervosum*, cytotoxicity, cancer cell lines, anti-HIV-1 reverse transcriptase

---

**INTRODUCTION**

The genus *Syzygium* (Myrtaceae) comprises approximately 1,200 species and is widely distributed in Africa, extending east to the Hawaiian Islands, and from India and China southwards to southern Australia and New Zealand. The centres of diversity are Southern Asia, South-east Asia, Malaysia, Australia and New Caledonia [1-4]. Many phytochemical substances from this genus have been isolated, e.g. terpenoids [5-7], triterpenes [8-11], phenylpropanoids [12-14], a macrocyclic ellagitannin [15], phenolic compounds [8, 16, 17], flavonoid glycosides [8, 17, 18], chalcones [11, 17, 19-21], flavanones [17, 19, 20], tannins [8] and lignins [8]. Some isolated compounds have been known to exhibit interesting biological properties including anti-HIV [6, 7], antibacterial [9], antimutagenic [12], antifungal [14], antitermitic [14], antioxidant [15-17],...
MATERIALS AND METHODS

Resources and Environment, Bangkok, Thailand. Herbarium, Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Plant

Merck silica gel 60H.

Agilent t reported in record on as follows: tetramethylsilane (TMS) and coupling constants (J values) in Hz. Peak multiplicities are indicated as follows: s (singlet), d (doublet), dd (doublet of doublet) and m (multiplet). Infrared spectra were recorded on an FT-IR model TENSOR 27 (Bruker) spectrometer and absorption frequencies were reported in reciprocal centimeters (cm⁻¹). Mass spectra (EI-MS) were performed with a GC/MS Agilent technologies system 6850 II/5973 using ionisation energy of 70 eV. High-resolution mass spectrometry (HRMS, electrospray ionisation mode) was measured on a Q-TOF-2™ (Waters) spectrometer. Flash column chromatography was performed employing Merck silica gel 60 and Merck silica gel 60H. Thin layer chromatography (TLC) was performed with Merck silica gel 60 F254 aluminium plates. All solvents used for extraction and isolation were distilled at their boiling point ranges prior to use.

Plant Material and Extraction

Seeds of S. nervosum were collected in July-August 2009 from Lamphun province, Thailand. The voucher specimen of the plant (BKF no. 187213) was deposited at the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand.
Air dried seeds (3,216.83 g) were extracted with 10 L of dichloromethane (2 x 3 days), followed by filtration. The filtrates were combined and evaporated *in vacuo* to give a dichloromethane extract (97.47 g).

**Isolation**

The isolation of compounds 1 and 2 was accomplished by using silica gel column chromatography with gradient elution. The dichloromethane extract (97.47 g) was fractionated by hexane-dichloromethane, dichloromethane-ethyl acetate and ethyl acetate-methanol respectively. All fractions were combined based on TLC profiles and evaporated to dryness *in vacuo* to give six fractions: F1-F6. F3 was further fractionated by repeated silica gel column chromatography into two subfractions to afford, after recrystallisation from CH₂Cl₂/hexane and EtOAc/hexane, compounds 1 (4.32 g) and 2 (1.18 g) respectively. In addition, 1 (3.48 g) and 2 (4.72 g) were also obtained from similar column chromatography of F4. The structure elucidation of 1 and 2 was achieved through comparison of their spectral data with previously reported data. The experimental data for the isolated compounds 1 and 2 are as follows.

**Compound 1**: Orange solid, mp 120.8-122.3°C; EI-MS *m/z*: 298 [M]+; HRMS (ESI) *m/z*: 321.1104, calc'd 321.1103 for C₁₅H₁₈O₄Na [M+Na]+; IR (CH₂Cl₂) (υmax, cm⁻¹): 3444, 2935, 1628, 1554, 1165; ¹H NMR (400 MHz, δ ppm, J/Hz): 2.14 (3H, s, 5'-CH₃), 2.16 (3H, s, 3'-CH₃), 3.66 (3H, s, 6'-OCH₃), 5.38 (1H, s, 4'-OH), 7.41 (3H, m, H-3,4,5), 7.64 (2H, m, H-2,6), 7.84 (1H, d, J = 15.7 Hz, H₉), 7.99 (1H, d, J = 15.7 Hz, H₀), 13.69 (1H, s, 2'-OH); ¹³C NMR (100 MHz, δ ppm): 7.6 (5'-CH₃), 8.2 (3'-CH₃), 62.3 (6'-OCH₃), 106.6 (C-1'), 109.0 (C-3'), 109.0 (C-5'), 126.7 (C₀), 128.4 (C-5), 128.4 (C-3), 128.9 (C-6), 128.9 (C-2), 130.2 (C-4), 135.3 (C-1'), 142.9 (C₀), 158.8 (C-6'), 159.3 (C-4'), 162.0 (C-2'), 193.4 (C=O).

**Compound 2**: Orange solid, mp 140.4-141.5°C; EI-MS *m/z*: 298 [M]+; HRMS (ESI) *m/z*: 321.1102, calc'd 321.1103 for C₁₅H₁₈O₄Na [M+Na]+; IR (CH₂Cl₂) (υmax, cm⁻¹): 3450, 2974, 1649, 1619, 1499, 1055; ¹H NMR (400 MHz, δ ppm, J/Hz): 1.40 (3H, s, 6-CH₃), 1.42 (3H, s, 6-CH₃), 1.86 (3H, s, 8-CH₃), 2.92 (1H, dd, J = 17.9, 3.7 Hz, H-3a), 3.03 (1H, dd, J = 17.9, 10.8 Hz, H-3b), 5.32 (1H, dd, J = 10.8, 3.7 Hz, H-2), 7.37-7.48 (5H, m, H-2',3,4',5,6'), 15.80 (1H, s, 4-OH); ¹³C NMR (100 MHz, δ ppm): 7.9 (8-CH₃), 23.1 (6-CH₃), 25.5 (6-CH₃), 38.2 (C-3), 52.3 (C-6), 76.0 (C-2), 101.4 (C-10), 107.3 (C-8), 125.8 (C-2',6'), 128.9 (C-3',4',5'), 138.0 (C-1'), 161.2 (C-9), 182.8 (C-4), 198.0 (C-7), 201.7 (C-5).

**Evaluation of Cytotoxic Activity**

The isolated compounds were submitted to preliminary cytotoxicity assays against cancer cell lines at the cytotoxicity test Service Centre at the Department of Microbiology, Mahidol University, Thailand. The cytotoxic activities of extracts and compounds were determined using the standard sulforhodamine B (SRB) assay in 96-well microtiter plates [36, 37]. Ellipticine was used as a positive control as it directly interacts with DNA, which is the ultimate target of cancer chemotherapy [38, 39]. Altogether, seven cell lines were employed: P-388 (mouse lymphoid neoplasma), KB (human epidermoid carcinoma in the mouth), HT29 (human colon cancer), MCF-7 (human breast cancer), Lu-1 (human lung cancer), A549 (human lung cancer) and ASK (rat glioma), together with Hek 293 (noncancerous human embryonic kidney cell). The cytotoxic activity is expressed as 50% effective dose (ED₅₀).
Anti-HIV-1 RT Assay

The isolated compounds were submitted to preliminary screening of inhibitory effect on HIV-1 RT at the cytotoxicity test Service Centre at the Department of Microbiology, Mahidol University, Thailand. The compounds were dissolved in DMSO at the concentration of 20 mg/mL and processed further to remove tannin. The assay was carried out in duplicate in a 96-well microtiter plate using the tannin-free supernatant of each compound as previously described [40]. An appropriate amount of HIV-1 RT (Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong) was employed and standardised with fagaronine chloride. This compound and nevirapine were used as positive controls, while DMSO was used as a negative control. The test compounds were prescreened at 200 μg/mL. The results from duplicate wells were averaged and the percentage of inhibition was calculated.

RESULTS AND DISCUSSION

Structure Elucidation

The results of analysis were compared with spectral data from previous reports [11, 19, 20, 33-35] and the structures of 1 and 2 were established as 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC) and (2S)-4-hydroxy-6,6,8-trimethyl-2-phenyl-2H-1-benzopyran-5,7-(3H,6H)-dione (hariganetin) respectively (Figure 1).

![Figure 1. Structures of DMC (1) and hariganetin (2)](image)

Compound 1 (orange solid) has a melting point of 120.8-122.3°C which is comparable to that reported in the literature, i.e. 125-126°C [19, 20]. The HRMS analysis shows [M+Na]⁺ ion at m/z 321.1104, indicating a molecular formula of C₁₅H₁₈O₄Na (calcd: 321.1103). The EI-MS exhibits a molecular ion peak at m/z 298 [M⁺], corresponding to a molecular formula of C₁₅H₁₈O₄ and fragmentation ions are found at m/z 221, 194, 166, 103 and 77, which is identical with the literature [11, 19, 20]. The IR spectrum shows a characteristic C=O stretching band of a conjugated carbonyl system at 1628 cm⁻¹. The absorption band at 3444 cm⁻¹ is assigned to a free hydroxyl as well as an H-bonded hydroxyl group. The ¹H NMR (400 MHz) spectrum of compound 1 (Table 1) exhibits signals for a monosubstituted phenyl group at δ 7.41 and 7.64, a trans-disubstituted double bond at δ 7.84 and 7.99 (JAB = 15.7 Hz) and two benzylic methyl groups at δ 2.14 and 2.16 ppm. Furthermore, the spectrum reveals the presence of three singlets for dihydroxy and methoxyl groups at δ 13.69 (2'-OH), 5.38 (4'-OH) and 3.66 (6'-OCH₃) ppm.

The complete structure of 1 was determined by analysing the 2D-NMR data including heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC) spectra. The HMQC spectrum allows us to connect the protons and carbons as shown in Table 1. The HMBC spectrum (Table 2) shows correlation of the proton signal at δ 7.84 (d, J = 15.7
Table 1. $^1$H-NMR (400 MHz) and $^{13}$C-NMR (100 MHz) spectra in CDCl$_3$ for isolated flavonoids 1 and 2

<table>
<thead>
<tr>
<th>Position</th>
<th>2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone (1)</th>
<th>Position</th>
<th>(2S)-4-Hydroxy-6,6,8-trimethyl-2-phenyl-2H-1-benzopyran-5,7-(3H,6H)-dione (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^1$H (J Hz)</td>
<td>$^{13}$C (DEPT)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>135.3 (C)</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>7.64 m</td>
<td>128.9 (CH)</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>7.41 m</td>
<td>128.4 (CH)</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>7.64 m</td>
<td>128.9 (CH)</td>
<td>5</td>
</tr>
<tr>
<td>$\beta$</td>
<td>7.84 d (15.7)</td>
<td>142.9 (CH)</td>
<td>7</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>7.99 d (15.7)</td>
<td>126.7 (CH)</td>
<td>8</td>
</tr>
<tr>
<td>CO</td>
<td>-</td>
<td>193.4 (C)</td>
<td>9</td>
</tr>
<tr>
<td>1'</td>
<td>-</td>
<td>106.6 (C)</td>
<td>10</td>
</tr>
<tr>
<td>2'</td>
<td>-</td>
<td>162.0 (C)</td>
<td>1'</td>
</tr>
<tr>
<td>3'</td>
<td>-</td>
<td>109.0 (C)</td>
<td>2'</td>
</tr>
<tr>
<td>4'</td>
<td>-</td>
<td>159.3 (C)</td>
<td>3'</td>
</tr>
<tr>
<td>5'</td>
<td>-</td>
<td>109.0 (C)</td>
<td>4'</td>
</tr>
<tr>
<td>6'</td>
<td>-</td>
<td>158.8 (C)</td>
<td>5'</td>
</tr>
<tr>
<td>2'-OH</td>
<td>13.69 s</td>
<td>-</td>
<td>6'</td>
</tr>
<tr>
<td>3'-CH$_3$</td>
<td>2.14 s</td>
<td>8.2 (CH$_3$)</td>
<td>4'-OH</td>
</tr>
<tr>
<td>4'-OH</td>
<td>5.38 s</td>
<td>-</td>
<td>6'-CH$_3$</td>
</tr>
<tr>
<td>5'-CH$_3$</td>
<td>2.16 s</td>
<td>7.6 (CH$_3$)</td>
<td>6'-CH$_3$</td>
</tr>
<tr>
<td>6'-OCH$_3$</td>
<td>3.66 s</td>
<td>62.3 (CH$_3$)</td>
<td>8'-CH$_3$</td>
</tr>
</tbody>
</table>

* $\delta$ in ppm from TMS [coupling constants (J) in Hz are given in parentheses.]

Table 2. $^1$H-$^{13}$C and $^1$H-$^1$H correlations for isolated flavonoids 1 and 2

<table>
<thead>
<tr>
<th>Position H</th>
<th>2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone (1)</th>
<th>Position H</th>
<th>(2S)-4-Hydroxy-6,6,8-trimethyl-2-phenyl-2H-1-benzopyran-5,7-(3H,6H)-dione (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMBC Correlation</td>
<td>COSY Correlation</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C-3, 4, $\beta$</td>
<td>H-3</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>C-1, 2, 5</td>
<td>H-2, 4</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>C-2, 3, 5, 6</td>
<td>H-3, 5</td>
<td>2'</td>
</tr>
<tr>
<td>5</td>
<td>C-1, 3, 6</td>
<td>H-4, 6</td>
<td>3'</td>
</tr>
<tr>
<td>6</td>
<td>C-2, 4, 5</td>
<td>H-5</td>
<td>4'</td>
</tr>
<tr>
<td>$\beta$</td>
<td>C-1, 2, 6, $\alpha$, CO</td>
<td>H-$\alpha$</td>
<td>5'</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>C-1, 1', $\beta$, CO</td>
<td>H-$\beta$</td>
<td></td>
</tr>
<tr>
<td>3'-CH$_3$</td>
<td>C-2', 3', 4'</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5'-CH$_3$</td>
<td>C-4', 5', 6'</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6'-OCH$_3$</td>
<td>C-6'</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Compound 2, isolated also as an orange solid, has a melting point of 140.4-141.5°C. It was determined as C_{18}H_{18}O_{4}Na by HRMS, showing the [M+Na]^+ ion at m/z 321.1102 (calcd: 321.1103). The EI-MS exhibits a molecular ion peak at m/z 298 [M]^+, corresponding to a molecular formula of C_{18}H_{18}O_{4}. The IR spectrum exhibits the C=O stretching of two carbonyl groups at 1619 and 1649 cm\(^{-1}\) and the carbonyl conjugated C=C stretching at 1449 cm\(^{-1}\). The absorption bands at 3450 and 1168 cm\(^{-1}\) are assigned to the O-H and C-O stretching respectively. The \(^1\)H NMR spectrum (Table 1) shows three methyl protons appearing as singlets at \(\delta\) 1.40, 1.42 and 1.86 ppm (\(9\)H, s, 6,6,8-CH\(_3\) respectively) and one hydroxyl proton at \(\delta\) 15.80 ppm (\(s\), 4-OH\(\)), hydrogen-bonded to the carbonyl group. The protons at the 3-position appear as a doublet-of-doublet at \(\delta\) 2.92 (\(J = 17.9, 3.7\) Hz) and 3.03 (\(J = 17.9, 10.8\) Hz) and the oxymethine proton H-2 shows a doublet-of-doublet signal at \(\delta\) 5.32 (\(J = 10.8, 3.7\) Hz) ppm. The \(^13\)C NMR spectrum displays several characteristic signals: the C-4 carbon with a hydroxyl group shows a signal at \(\delta\) 182.8 ppm; the two carbonyl carbons C-5 and C-7 show peaks at \(\delta\) 201.7 and 198.0 ppm respectively; and the endocyclic double bond at C-9 shows a unique signal at \(\delta\) 161.2 ppm.

Furthermore, 2D-NMR spectra, HMQC and HMBC were also recorded for compound 2, as shown in Table 2, to confirm the structure. The HMQC data are used to explain the correlation between \(^1\)H and \(^13\)C NMR spectra. They display correlations such as the H-2 proton being coupled by C-2 carbon, the H-3 proton being associated with C-3 carbon, and the three methyl protons showing correlations with 8-CH\(_3\) and 6-(CH\(_3\))\(_2\) carbons at \(\delta\) 7.9, 23.1 and 25.5 ppm respectively.

The HMBC spectrum shows correlations of the proton signals at \(\delta\) 2.92 and 3.03 of the methylene protons at H-3 with the carbon signals at \(\delta\) 76.0 (C-2), 182.8 (C-4), 101.4 (C-10) and 138.0 (C-1'); the proton signal at \(\delta\) 5.32 (H-2) correlates with the carbon signals at \(\delta\) 38.2 (C-3), 182.8 (C-4), 138.0 (C-1') and 125.8 (C-2', 6'); the proton signal at \(\delta\) 1.86 (\(s\), 8-CH\(_3\)) shows association with the carbon signals at \(\delta\) 198.0 (C-7), 107.3 (C-8) and 161.2 (C-9); and the proton signals of the dimethyl group at \(\delta\) 1.40 and 1.42 (\(s\), 6-CH\(_3\)) show correlation with the carbon signals at \(\delta\) 201.7 (C-5), 52.3 (C-6) and 198.0 (C-7). These spectral analyses were compared to the literature [33-35] and the structure is established to be hariganetin (2).

**Biological Activities**

DMC (1) has been reported regarding its biological properties such as antibiotic [41], antitumour [42-44], anticancer [21], antiprotozoal [45] and cytotoxic [17, 46, 47] activities. In addition, it shows an inhibitory effect on the viral neuraminidases from two influenza viral strains, H1N1 and H9N2 [48]. In our investigation the two isolated flavonoids were preliminarily evaluated for anticancer and anti-HIV-1 RT activities (Table 3). DMC (1) shows recordable cytotoxicity to P-388, KB, HT29, MCF-7, A549, ASK and Hek293 cell lines, while hariganetin (2) is inactive. However, both compounds (1 and 2) exhibit an anti-HIV-1 RT activity, with an inhibition of 67.5% and 64.1% respectively.
Table 3. Cytotoxicity and anti-HIV-1 RT activities of isolated flavonoids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxicity (ED50, μg/mL)</th>
<th>HIV-1 RT assay % Inhibition at 200 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-388</td>
<td>KB</td>
</tr>
<tr>
<td>DMC (1)</td>
<td>10.31</td>
<td>15.90</td>
</tr>
<tr>
<td>Hariganetin (2)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Ellipticine (Positive control)</td>
<td>0.56</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Notes: ED50 less than 20 μg mL−1 for extract and less than 4 μg mL−1 for pure compound are considered active. P-388 = murine lymphocytic leukemia, KB = human oral nasopharyngal carcinoma, HT29 = human colon cancer, MCF-7 = human breast cancer, A549 = human lung cancer, ASK = rat glioma cell, HeK293 = noncancerous human embryonic kidney cell. Ellipticine, an anticancer drug, was used as a positive control in the cytotoxicity test. NR = no response (ED50>20 μg mL−1), NT = not tested.

ACKNOWLEDGEMENTS

This research was supported by Chiang Mai University Mid-Career Research Fellowship Programme and Centre of Excellence for Innovation in Chemistry (PERCH-CIC). The Research Administration Centres of Chiang Mai University and North-Chiang Mai University are also thanked for financial support.

REFERENCES

1. L. A. Craven, “Unravelling knots or plaiting rope: What are the major taxonomic strands in Syzygium sens.lat. (Myrtaceae) and what should be done with them?”, Proceedings of 4th Flora Malesiana Symposium, 2001, Kuala Lumpur, Malaysia, pp.75-85.


24. N. Nuengchamnong and K. Ingkaninan, “On-line characterization of phenolic antioxidants in fruit wines from family myrtaceae by liquid chromatography combined with electrospray


© 2017 by Maejo University, San Sai, Chiang Mai, 50290 Thailand. Reproduction is permitted for noncommercial purposes.