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## Cytotoxic homoisoflavonoids from the bulbs of *Bellevalia flexuosa*

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### Abstract

Four new homoisoflavonoids, 7-*O*-methyl-8-demethoxy-3'-hydroxy-3,9-dihdropunctatin (**4**), 6-hydroxy-8-demethoxy-4'-*O*-methyl-3,9-dihdropunctatin (**8**), 7,4'-*O*-dimethyl-8-demethoxy-3,3'-dihydroxy-3,9-dihdropunctatin (**13**), and 7-*O*-methyl-3-hydroxy-3,9-dihdropunctatin (**14**) were identified from a chloroform extract of the bulbs of *Bellevalia flexuosa*, along with 13 known analogues. The structures were determined by analysis of HRMS and NMR data, while ECD spectroscopy enabled the assignment of the absolute configurations of the new compounds **4**, **8**, **13** and **16**. The cytotoxic activities of the isolated compounds (**1–17**) were evaluated using a panel of human cancer cell lines. Compounds **2** and **7** were the most potent against the MDA-MB-435 (melanoma) cancer cell line with IC<sub>50</sub> values of 1.6 and 2.0 μM, respectively, and were essentially equipotent against the OVCAR3 (ovarian) cancer cell line with IC<sub>50</sub> values of 9.5 and 10.8 μM,

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### Conflict of interest

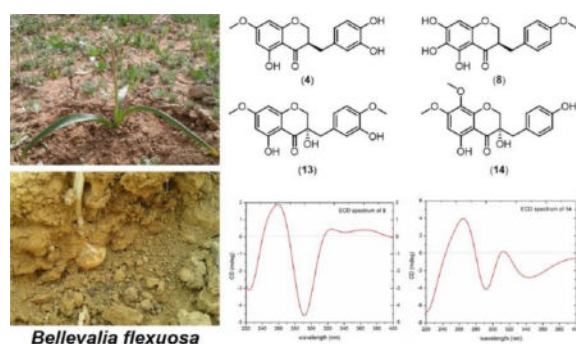
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### Appendix A. Supplementary data

Supplementary data to this article can be found online at

respectively. However, compound **7**, with an  $IC_{50}$  value of 3.6  $\mu$ M, was the most potent against the MDA-MB-231 (breast) cancer cell line.

## Graphical abstract



## Keywords

Homoisoflavonoids; *Bellevalia*; Bulbs; Absolute configuration; Cytotoxicity; Human cancer cell lines

## 1. Introduction

Homoisoflavonoids are a rare subclass of flavonoids possessing an extra carbon atom in their skeleton [1–5]. Biosynthetically, chalcones are thought to be the precursors of homoisoflavonoids [3]. Currently, about 250 natural homoisoflavonoids have been reported in the literature, with the majority being isolated from several genera of Asparagaceae and Fabaceae [1–4, 6, 7]. Based on their structures, homoisoflavonoids have been classified into five groups: 3-benzyl-4-chromanones, 3-benzyl-3-hydroxy-4-chromanones, 3-benzylidene-4-chromanones (*E* or *Z*), 3-benzylchrom-2-en-4-ones, and the scillascillins [4]. Homoisoflavonoids have attracted attention because of their various biological activities, including antioxidant, anti-inflammatory, antimutagenic, antimicrobial, antiallergic and antihistaminic, anti-diabetic, cytotoxic, and anti-angiogenic effects, as well as protein tyrosine kinase inhibition activity [3, 4].

Although Jordan is a small country of about 96,188 km<sup>2</sup>, it has a remarkable diversity of wildlife, which could be attributed to its unique location at the intersection of three continents, encapsulating four bio-geographical zones: Mediterranean, Irano-Turanian, Saharo-Arabian, and Tropical (Sudanian penetration) [8–10]. Within these different zones, thirteen vegetation types are identified, reflecting Jordan's diverse landscape, climate, and geology [8, 11]. More than 2,600 vascular plant species belonging to 113 family and 810 genera are reported to grow in the wild, of which 100 are endemic and more than 70 species are considered extinct [8]. Hence, studies of Jordan's wild plants have been initiated [12–14] as a potential source of drug leads.

In the current study, the bulbs of *Bellevalia flexuosa* Boiss. (Asparagaceae) have been explored. *B. flexuosa*, which is known as “Common Roman Squill”, and in Jordan as

“Drooping Onion” [15], is one of eleven species that are reported by Al-Eisawi to grow in Jordan in the wild [16]. Although no medical use is reported for this plant species by the local people of Jordan, a closely related species (*B. eigi*) was investigated recently, yielding a set of homoisoflavonoids [6]. *B. flexuosa* is a perennial herb with underground bulbs that is found flowering from February to March. It flourishes in mountains and waste grounds [16]. Chemical investigation of the chloroform extract of the bulbs of *B. flexuosa* resulted in the isolation and identification of seventeen homoisoflavonoids; of which thirteen were known (**1–3**, **5–7**, **9–12**, and **15–17**) and four were new analogues: 7-*O*-methyl-8-demethoxy-3'-hydroxy-3,9-dihdropunctatin (**4**), 6-hydroxy-8-demethoxy-4'-*O*-methyl-3,9-dihdropunctatin (**8**), 7,4'-*O*-dimethyl-8-demethoxy-3,3'-dihydroxy-3,9-dihdropunctatin (**13**), and 7-*O*-methyl-3-hydroxy-3,9-dihdropunctatin (**14**). ECD spectroscopy was used to assign the absolute configurations of the new compounds **4**, **8**, **13**, and **14**.

Homoisoflavonoids were reported to have a broad range of biological activities, including cytotoxic effects [3]. Therefore, the isolated compounds (**1–17**) were tested for their cytotoxicity using three human cancer cell lines, namely MDA-MB-435 (melanoma), MDA-MB-231 (breast), and OVCAR3 (ovarian).

## 2. Experimental

### 2.1. General experimental procedures

Optical rotations, UV data, and ECD spectra were obtained using a Rudolph Research Autopol III polarimeter (Rudolph Research Analytical), a Varian Cary 100 Bio UV-vis spectrophotometer (Varian Inc.), and an Olis DSM 17 ECD spectrophotometer (Olis, Inc.). NMR data were collected using either a JEOL ECA-500 NMR spectrometer operating at 500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$  (JEOL Ltd.) or an Agilent 700 MHz NMR spectrometer (Agilent Technologies), equipped with a cryoprobe, operating at 700 MHz for  $^1\text{H}$  and 175 MHz for  $^{13}\text{C}$ . Residual solvent signals were utilized for referencing. HRMS data were acquired using a Thermo QExactive Plus mass spectrometer equipped with an electrospray ionization source (Thermo Fisher Scientific). Gemini-NX  $\text{C}_{18}$  analytical (5  $\mu\text{m}$ ; 250  $\times$  4.6 mm) and preparative (5  $\mu\text{m}$ ; 250  $\times$  21.2 mm) columns (both from Phenomenex) along with Atlantis T3  $\text{C}_{18}$  analytical (5  $\mu\text{m}$ ; 250  $\times$  4.6 mm) and semipreparative (5  $\mu\text{m}$ ; 250  $\times$  10.0 mm) columns (both from Waters Corp.) were used on a Varian Prostar HPLC system equipped with ProStar 210 pumps and a Prostar 335 photodiode array detector (PDA), with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2, Varian Inc.). Flash chromatography was performed on a Teledyne ISCO CombiFlash Rf 200 using Silica Gold columns (both from Teledyne Isco) and monitored by UV and evaporative light-scattering detectors. All other reagents and solvents were obtained from Fisher Scientific and were used without further purification.

### 2.2. Plant material

Bulbs of *B. flexuosa* were collected by Mohammad Bashabshah during flowering stage from the campus of the Jordan University of Science and Technology (JUST), Irbid, Jordan in March/April 2016. The plant material was identified by Mohammad Al-Gharaibeh, Plant Taxonomist, Faculty of Agriculture, JUST. A voucher specimen (PHS-122) was deposited in

the herbarium of the Faculty of Pharmacy, JUST. The bulbs were cleaned of mud, sliced into small pieces, and air dried at rt in a well-ventilated area.

### 2.3. Extraction and isolation

Air-dried bulbs of *B. flexuosa* were ground to a powder using a laboratory mill. The powdered bulbs (850 g) were extracted exhaustively with  $\text{CHCl}_3$  by soaking at rt. The solvent was evaporated under reduced pressure to yield the  $\text{CHCl}_3$  extract (3.1 g), which was reconstituted in a 500 mL mixture of 5:4:1  $\text{H}_2\text{O}:\text{CHCl}_3:\text{MeOH}$ . The mixture was stirred for 30 min and left to separate in a separatory funnel. The organic layer was collected and evaporated to dryness under reduced pressure. The dried organic extract was reconstituted in 200 mL of 1:1  $\text{MeOH}:\text{CH}_3\text{CN}$  and 200 mL of hexanes and transferred into a separatory funnel. The biphasic solution was shaken vigorously. The  $\text{MeOH}/\text{MeCN}$  layer was evaporated to dryness under vacuum. The dried  $\text{MeOH}/\text{CH}_3\text{CN}$  layer (2.1 g) was dissolved in  $\text{CHCl}_3$  and mixed with Celite 545. Normal-phase flash chromatography was run using a gradient solvent system of hexanes- $\text{CHCl}_3$ - $\text{MeOH}$ , at a flow rate of 35 mL/min, and column volumes of 41.3 over a total run time of 30.7 min, to yield seven fractions. Fraction 3 (267.9 mg) was subjected to preparative HPLC over a Gemini column using a gradient system of 50:50 to 60:40 of  $\text{MeOH}-\text{H}_2\text{O}$  (0.1% formic acid) over 30 min at a flow rate of 20 mL/min to yield 8 subfractions, of which subfraction 3 was identified as compound **15** (2.1 mg). Subfraction 7 (1.9 mg) was subjected to semipreparative HPLC purification over Atlantis T3 column using a gradient solvent system of 40:60 to 50:50  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$  (0.1% formic acid) over 15 min at a flow rate of 4.6 mL/min to yield compounds **5** (0.7 mg) and **13** (0.4 mg). Subfraction 8 (8.3 mg) was subjected to semipreparative HPLC using Atlantis T3 column and a gradient solvent system of 40:60 to 60:40  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$  (0.1% formic acid) over 20 min at a flow rate of 4.6 mL/min to yield compounds **7** (5.4 mg) and **13** (0.3 mg).

Fraction 4 (544.7 mg) was subjected to preparative HPLC over a Gemini column using a gradient system of 60:40 to 70:30 of  $\text{MeOH}-\text{H}_2\text{O}$  (0.1% formic acid) over 20 min at a flow rate of 21.2 mL/min to yield compound **5** (335.6 mg).

Fraction 5 (116.0 mg) was subjected to preparative HPLC over a Gemini column using a gradient system of 50:50 to 70:30 of  $\text{MeOH}-\text{H}_2\text{O}$  (0.1% formic acid) over 20 min at a flow rate of 21.2 mL/min to yield seven sub-fractions. Further HPLC purification of subfractions 3 (3.4 mg) and 4 (3.2 mg) using semipreparative HPLC (Atlantis T3 column) and a gradient solvent systems of 40:60 to 50:50  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$  (0.1% formic acid) over 15 min at a flow rate of 4.6 mL/min yielded compounds **17** (1.3 mg) and **2** (2.1 mg) from subfractions 3 and 4, respectively. Subfractions 6 (2.2 mg) and 7 (14.3 mg) were purified using semipreparative HPLC (Atlantis T3 column) and a gradient solvent system of 40:60 to 60:40  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$  (0.1% formic acid) over 20 min at a flow rate of 4.6 mL/min to yield compounds **4** (0.9 mg) and **16** (10.8 mg) from subfractions 6 and 7, respectively.

Fraction 6 (379.3 mg) was purified using preparative HPLC (Gemini column) and a gradient solvent system of 50:50 to 55:45 of  $\text{MeOH}-\text{H}_2\text{O}$  (0.1% formic acid) over 20 min, hold for 5 min and then increasing to 60:40 over 10 min at a flow rate of 21.2 mL/min to yield 5 subfractions. Subfraction 1 (13.2 mg) was further purified using semipreparative (Atlantis T3 column) and a gradient solvent system of 30:70 to 40:60  $\text{CH}_3\text{CN}-\text{H}_2\text{O}$  (0.1% formic

acid) over 15 min at a flow rate of 4.6 mL/min to yield compound **8** (2.3 mg) and another subfraction that was further purified using semipreparative HPLC method over Atlantis T3 column and an isocratic solvent system of 28:72 CH<sub>3</sub>CN-H<sub>2</sub>O (0.1% formic acid) for 50 min at a flow rate of 4.6 mL/min to yield compounds **9** (2.3 mg) and **14** (1.9 mg). Subfractions 2 (15.6 mg) and 3 (39.0 mg) were purified using semipreparative HPLC (Atlantis T3 column) and a gradient solvent systems of 40:60 to 60:50 CH<sub>3</sub>CN-H<sub>2</sub>O (0.1% formic acid) over 15 min at a flow rate of 4.6 mL/min to yield compound **6** (9.1 mg) from subfraction 2 and compounds **3** (1.4 mg), **6** (1.9 mg), and **10** (19.2 mg) from subfraction 3. Subfractions 4 (3.4 mg) and 5 (3.8 mg) were purified using semipreparative HPLC (Atlantis T3 column) and gradient solvent systems of 40:60 to 50:50 CH<sub>3</sub>CN-H<sub>2</sub>O (0.1% formic acid) over 15 min at a flow rate of 4.6 mL/min to yield compounds **1** (0.5 mg) and **10** (0.5 mg) from fraction 4 and compounds **2** (0.9 mg), **5** (2.3 mg), **11** (0.9 mg), and **12** (1.5 mg) from subfraction 5.

**2.3.1. 7-O-Methyl-8-demethoxy-3'-hydroxy-3,9-dihydropunctatin (4)**—Yellowish oil;  $[\alpha]_D^{28} - 27(c0.1, \text{MeOH})$ ; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 334 (3.23), 288 (3.98), 220 (3.94) nm; ECD ( $c 0.9 \times 10^{-4}$  M, MeOH)  $\lambda ( \epsilon)$  244 (+0.68) nm, 269 (+0.96) nm, 295 (−2.07), 320 (+0.18) nm (Fig. 3); HRESIMS  $m/z$  317.1016 [M + H]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>17</sub>O<sub>6</sub>, 317.1020).

**2.3.2. 6-Hydroxy-8-demethoxy-4'-O-methyl-3,9-dihydropunctatin (8)**—Light yellow amorphous powder;  $[\alpha]_D^{28} - 36(c0.1, \text{MeOH})$ ; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 360 (3.32), 291 (4.05), 242 (3.94), 229 (3.99) nm; ECD ( $c 0.9 \times 10^{-4}$  M, MeOH)  $\lambda ( \epsilon)$  260 (+2.02) nm, 293 (−4.79), 323 (+0.50) nm (Fig. 3); HRESIMS  $m/z$  317.1016 [M + H]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>17</sub>O<sub>6</sub>, 317.1020).

**2.3.3. 7,4'-O-dimethyl-8-demethoxy-3,3'-dihydroxy-3,9-dihydropunctatin (13)**—White amorphous powder;  $[\alpha]_D^{28} - 96(c0.05, \text{MeOH})$ ; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 334 (3.35), 288 (4.08), 218 (4.10) nm; ECD ( $c 0.9 \times 10^{-4}$  M, MeOH)  $\lambda ( \epsilon)$  253 (+1.80) nm, 295 (−3.52), 320 (−0.53) nm (Fig. 3); HRESIMS  $m/z$  347.1122 [M + H]<sup>+</sup> (calcd for C<sub>18</sub>H<sub>19</sub>O<sub>7</sub>, 347.1125).

**2.3.4. 7-O-methyl-3-hydroxy-3,9-dihydropunctatin (14)**—Light yellow amorphous powder;  $[\alpha]_D^{28} - 170(c0.1, \text{MeOH})$ ; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 348 (3.58), 292 (4.15), 224 (4.15) nm; ECD ( $c 0.9 \times 10^{-4}$  M, MeOH)  $\lambda ( \epsilon)$  265 (+4.16) nm, 292 (−4.53), 313 (+0.42) nm (Fig. 3); HRESIMS  $m/z$  347.1124 [M + H]<sup>+</sup> (calcd for C<sub>18</sub>H<sub>19</sub>O<sub>7</sub>, 347.1125).

## 2.4. Cytotoxicity assay

Compounds (**1–17**) were tested for cytotoxicity against human melanoma cancer cells MDA-MB-435 [17], human breast cancer cells MDA-MB-231, and human ovarian cancer cells OVCAR3 as described previously [18, 19]. Briefly, the cell lines were propagated at 37 °C in 5% CO<sub>2</sub> in RPMI 1640 medium, supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells in log phase growth were harvested by trypsinization followed by two washings to remove all traces of enzyme. A

total of 5,000 cells were seeded per well of a 96-well clear, flat-bottom plate (Microtest 96<sup>®</sup>, Falcon) and incubated overnight (37 °C in 5% CO<sub>2</sub>). Samples dissolved in DMSO were then diluted and added to the appropriate wells. The cells were incubated in the presence of test substance for 72 h at 37 °C and evaluated for viability with a commercial absorbance assay (CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay, Promega Corp, Madison, WI) that measured viable cells. IC<sub>50</sub> values are expressed in μM relative to the solvent (DMSO) control. Taxol was used as a positive control.

### 3. Results and discussion

Air-dried bulbs of *B. flexuosa* were extracted with CHCl<sub>3</sub>, and the resulting crude extract was reconstituted in a mixture of 5:4:1 H<sub>2</sub>O:CHCl<sub>3</sub>:MeOH. The dried organic extract was reconstituted in 1:1 MeOH/CH<sub>3</sub>CN and then defatted using hexanes. The resulting dried MeOH/CH<sub>3</sub>CN extract was fractionated using normal phase flash chromatography. HPLC methods, preparative and semipreparative, were used for purifications of the fractions leading to the isolation of seventeen homoisoflavonoids (**1–17**) (Fig. 1).

The structures of thirteen known homoisoflavonoids analogues (**1–3**, **5–7**, **9–12**, and **15–17**) were established by comparison of NMR (1D/2D), HRMS, and ECD data with literature values and were identified as: 4',5,7-trihydroxyhomisoflavanone (**1**) [6], 3'-hydroxy-3,9-dihydroeucomin (**2**) [6], 3,9-dihdropunctatin (**3**) [20], 7-*O*-methyl-3,9-dihdropunctatin (**5**) [6], 8-*O*-demethyl-7-*O*-methyl-3,9-dihdropunctatin (**6**) [6], 7,4'-di-*O*-methyl-3'-hydroxy-3,9-dihdropunctatin (**7**) [6], 6-hydroxy-7-*O*-methyl-3,9-dihdropunctatin (**9**) [6], 7-*O*-methyl-3'-hydroxypunctatin (**10**), 3-(4-hydroxybenzyl)-5-hydroxy-6,7,8-trimethoxychroman-4-one (**11**) [21], 7-*O*-methyl-8-demethoxy-3-hydroxy-3,9-dihdropunctatin (**12**) [6], 5-hydroxy-7,8-dimethoxychroman-4-one (**15**) [6], 7-*O*-methylpunctatin (16) [6], and isomuscumisin (**17**) [6], (Figs. S1–S3, S5–S7, S9–S12, S15–S17, Supplementary Data)

Compound **4** (0.93 mg) was isolated as a yellowish oil with a molecular formula of C<sub>17</sub>H<sub>16</sub>O<sub>6</sub> (10 degrees of unsaturation) as determined by HRESIMS (*m/z* 317.1016 [M + H]<sup>+</sup>, calcd. 317.1020), which was further supported by <sup>1</sup>H, <sup>13</sup>C, and edited HSQC NMR data (Tables 1 and 2, Fig. S4, Supplementary Data). Compound **4** showed characteristic NMR signals indicative of a 3-benzylchroman-4-one homoisoflavonoid analogue with very high structural similarity to **2**, both sharing the same molecular formula. HMBC data analysis indicated the structure of **4** to be different from that of **2** by the methylation position. HMBC correlations from H<sub>3</sub>-7' (δ<sub>H</sub> 3.88, s) to C-4' (δ<sub>C</sub> 145.5) in **2** and from H<sub>3</sub>-10 (δ<sub>H</sub> 3.81, s) to C-7 (δ<sub>C</sub> 168.0) in **4** enabled the assignment of **2** as a 4'-*O*-methyl analogue and **4** as a 7-*O*-methyl analogue. In the latter, HMBC correlations from H-8 (δ<sub>H</sub> 5.97, d, *J* = 2.3) to C-4a (δ<sub>C</sub> 102.7), C-6 (δ<sub>C</sub> 95.1), C-7 (δ<sub>C</sub> 168.0), and C-8a (δ<sub>C</sub> 163.0); from H-6 (δ<sub>H</sub> 6.06, d, *J* = 2.3) to C-5 (δ<sub>C</sub> 164.6), C-7, C-8 (δ<sub>C</sub> 94.0), and C-4a, from 5-OH (δ<sub>H</sub> 12.11, s) to C-4a, C-5, and C-6 established the substitution pattern of ring A. In addition, HMBC correlations from H-2' (δ<sub>H</sub> 6.75, d, *J* = 1.3) to C-9 (δ<sub>C</sub> 32.3), C-4' (δ<sub>C</sub> 142.4), and C-6' (δ<sub>C</sub> 121.8); from H-5' (δ<sub>H</sub> 6.81, d, *J* = 7.9) to C-1' (δ<sub>C</sub> 131.0) and C-3' (δ<sub>C</sub> 143.8); from H-6' (δ<sub>H</sub> 6.66, dd, *J* = 7.9, 1.3) to C-9, C-2' (δ<sub>C</sub> 116.3), and C-4' established the substitution pattern of ring C. Further examination of the 2D NMR data established the structure of **4** (Fig. 2) as 7-*O*-



methyl-8-demethoxy-3'-hydroxy-3,9-dihydropunctatin. The absolute configurations of 3-benzylchroman-4-one-type homoisoflavonoids are assigned using electronic circular dichroism (ECD) spectroscopy [22], in which a negative Cotton effect in the 287–295 nm region of the ECD curves is indicative of a *3R* configuration [22]. Compound **4** showed a negative Cotton effect at 295 nm ( $\epsilon = -2.07$ ) in the ECD spectrum, supporting an *R*-configuration at C-3 (Fig. 3).

HRESIMS and NMR data of compound **8** (2.3 mg), which was obtained as a yellow amorphous powder, revealed its molecular formula as  $C_{17}H_{16}O_6$  ( $m/z$  317.1016  $[M + H]^+$ , calcd 317.1020) (Tables 1 and 2, Fig. S8, Supplementary Data). As in compound **4**, the NMR data of **8** showed characteristic signals indicative of a 3-benzylchroman-4-one-type homoisoflavonoid with structural similarity to **3** as they shared the same molecular formula. However, the 8-methoxy group of ring A ( $\delta_H/\delta_C$  3.95/61.7 for H<sub>3</sub>-11/C-11) in **3** was replaced by an aromatic proton singlet ( $\delta_H/\delta_C$  6.05/91.1 for H-8/C-8) in **8**. In addition, the aromatic proton singlet ( $\delta_H/\delta_C$  6.13/96.0 for H-6/C-6) in **3** was replaced by an exchangeable proton singlet ( $\delta_H$  5.03 for 6-OH) in **8**. Moreover, the exchangeable proton singlet in **3** ( $\delta_H$  4.75 for 4'-OH) was replaced by a methyl group ( $\delta_H/\delta_C$  3.91/56.5 for H<sub>3</sub>-7'/C-7'). The structure of **8** was inferred by further analysis of HMBC data. HMBC correlation from H-8 to C-4 (198.8), C-4a (102.5), C-6 (156.1), and C-8a (154.8) established the substitution pattern of ring A, while the HMBC correlation from H<sub>3</sub>-7' ( $\delta_H$  3.91, s) to C-4' ( $\delta_C$  154.5) confirmed the methylation position in **8**. Further analysis of the 2D NMR data established the structure of **8** to which the trivial name 6-hydroxy-8-demethoxy-4'-*O*-methyl-3,9-dihydropunctatin was assigned (Fig. 2). A negative Cotton effect at 293 nm in the ECD spectrum of compound **8** ( $\epsilon = -4.79$ ) indicated an *R*-configuration at C-3 (Fig. 3).

Compound **13** (0.66 mg) was obtained as a white amorphous powder. HRESIMS ( $m/z$  347.1122  $[M + H]^+$ , calcd 347.1125) NMR data indicated a molecular formula of  $C_{18}H_{18}O_7$  (Tables 1 and 2, Fig. S13, Supplementary Data). The NMR data indicated compound **13** as a 3-benzyl-3-hydroxychroman-4-one homoisoflavonoid analogue. The compound showed high structural similarity to **12**. However, compound **13** showed a methoxy group, as indicated by  $^1H$  and  $^{13}C$  NMR data ( $\delta_H/\delta_C$  3.88/56.0) and a hydroxyl group at  $\delta_H$  5.58 (3'-OH) consistent with the 30 amu difference in the HRMS data of **13** relative to **12**. The aromatic A<sub>2</sub>B<sub>2</sub> system of ring B in **12** was replaced by an ABM spin system in **13** ( $\delta_H$  6.82, d,  $J = 2.1$ ; 6.76, d,  $J = 8.1$ ; and 6.65, dd,  $J = 8.1, 2.1$ , for H-2', H-5', and H-6', respectively), indicating the presence of a 1,3,4-trisubstituted benzene ring. An HMBC correlation from the 4'-OCH<sub>3</sub> protons to C-4' ( $\delta_C$  145.9) confirmed its connectivity. The structure of compound **13** was deduced by further inspection of the 2D NMR data, including COSY and HMBC spectra (Fig. 2). Compound **13** was given the trivial name 7,4'-*O*-dimethyl-8-demethoxy-3,3'-dihydroxy-3,9-dihydropunctatin. A negative Cotton effect ( $\epsilon = -3.52$ ) at 297 nm of the ECD spectrum of **13** indicated an *S*-configuration at C-3 (Fig. 3) [22].

The HRESIMS ( $m/z$  347.1124  $[M + H]^+$ , calcd 347.1125) and NMR data of compound **14** (2.38 mg), which was obtained as a light yellow amorphous powder, indicated a molecular formula of  $C_{18}H_{18}O_7$  (Tables 1 and 2, Fig. S14, Supplementary Data). The NMR data of **14** showed distinctive peaks indicative of a 3-benzyl-3-hydroxychroman-4-one

homoisoflavonoid analogue. The compound showed high structural similarity to **12**. However, compound **14** had an extra methoxy group, as indicated by  $^1\text{H}$  and  $^{13}\text{C}$  NMR data ( $\delta_{\text{H}}/\delta_{\text{C}}$  3.83/61.7), consistent with the 30 amu difference in the HRMS data of **14** relative to **12**, replacing the aromatic doublet ( $\delta_{\text{H}}$  6.05, d,  $J = 2.1$  for H-8) in **12**. HMBC correlations from H-6 to C-4 (198.6), C-4a (100.4), C-5 (160.0), C-7 (162.3), and C-8 (129.9) confirmed the substitution pattern of ring A. The structure of compound **14** was deduced by further inspection of the 2D NMR data, including COSY and HMBC spectra (Fig. 2). Compound **14** was given the trivial name 7-*O*-methyl-3-hydroxy-3,9-dihydropunctatin. A negative cotton effect ( $\epsilon = -4.53$ ) at 292 nm in the ECD spectrum of **14** was indicative of an *S*-configuration at C-3 (Fig. 3) [22].

Compounds (**1–17**) were evaluated for their cytotoxic activities against the MDA-MB-435 (melanoma), MDA-MB-231 (breast), and OVCAR3 (ovarian) cancer cell lines. Compounds **2** and **7** were the most potent on the three cancer cell lines with  $\text{IC}_{50}$  values of 1.6, 14.2, 9.5 and 2.0, 3.6, and 10.8  $\mu\text{M}$ , respectively (Table 4). Compound **2** was reported to be active against colon cancer (HT-29 cell line  $\text{ED}_{50} = 2.78 \mu\text{M}$ ) and breast cancer (MDA-MB-435 cell line  $\text{ED}_{50} = 1.33 \mu\text{M}$ ). Compounds **2** and **7** were reported previously by our group to be active against breast cancer (MDA-MB-435 cell line) with  $\text{IC}_{50}$  values of 1.0 and 1.1  $\mu\text{M}$ , respectively [6]. Moreover, compound **7** showed moderate activity when tested against colon cancer (HT-29 cell line  $\text{IC}_{50} = 17.3 \mu\text{M}$ ) [6]. The cytotoxicity data of the new and known analogues isolated in the current work expanded our understanding of the structure-activity relationships of this unique class of flavonoids. The substitution pattern of ring B affects the activity significantly. Compound **1**, with a 4'-OH group, was inactive. However, compound **4**, with 3',4'-dihydroxy substituents, showed  $\text{IC}_{50}$  values of 14.3, 17.5, and 24.8  $\mu\text{M}$  against the MDA-MB-435, MDA-MB-231, and OVCAR3 cancer cell lines, respectively. Moreover, reducing the polarity of the ring B substituents results in improved cytotoxic activity. For example, the activity of compound **2**, with a 4'-methoxy substituent, increased by ~9- and 3-folds the activity against MDA-MB-435 and OVCAR3 cancer cell lines in comparison with compound **4** via  $\text{IC}_{50}$  values of 1.6 and 9.5  $\mu\text{M}$ , respectively. Similarly, although compound **10** was inactive, compound **7**, with a 4'-methoxy substituent, showed  $\text{IC}_{50}$  values of 2.0, 3.6, and 10.8  $\mu\text{M}$  against the MDA-MB-435, MDA-MB-231, and OVCAR3 cancer cell lines, respectively. The same pattern can be noticed when comparing the cytotoxic activities of compounds **12** and **13**. Compound **12** with a 4'-OH substituent was inactive, however compound **13**, with 3'-hydroxy and 4'-methoxy substituents, showed activity against the MDA-MB-435 cancer cell line with  $\text{IC}_{50}$  value of 14.4  $\mu\text{M}$ . In addition, introducing an OH group at C-3 increases the activity significantly, although compound **5** was inactive, compound **14**, with a 3-OH substituent, showed  $\text{IC}_{50}$  value of 15.2  $\mu\text{M}$  against the MDA-MB-435 cancer cell line.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



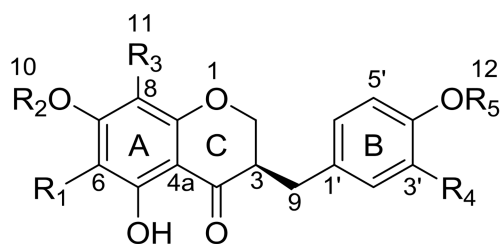
## Acknowledgments

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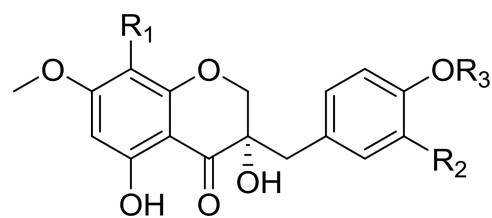
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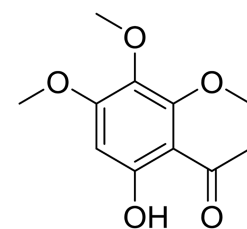
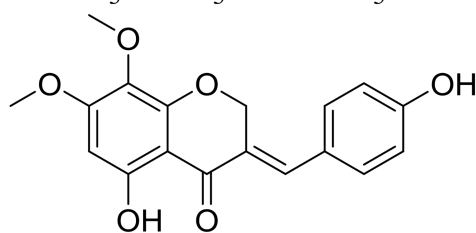
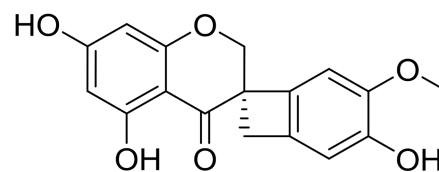
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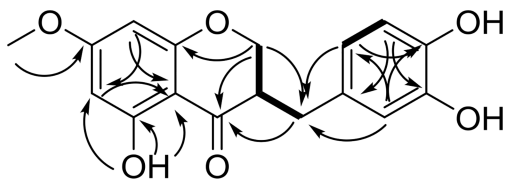
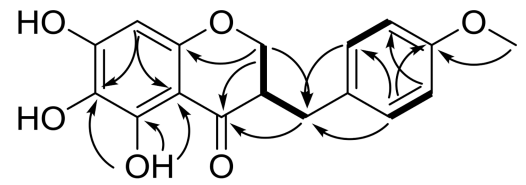
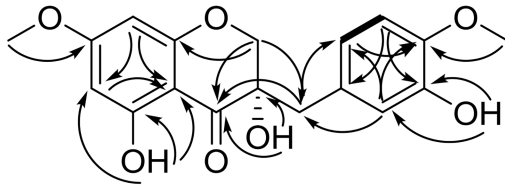
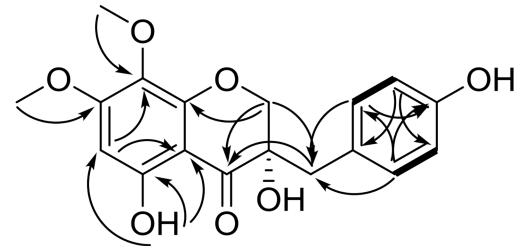
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<b>1:</b>	H	H	H	H	H
<b>2:</b>	H	H	H	OH	CH <sub>3</sub>
<b>3:</b>	H	H	OCH <sub>3</sub>	H	H
<b>4:</b>	H	CH <sub>3</sub>	H	OH	H
<b>5:</b>	H	CH <sub>3</sub>	OCH <sub>3</sub>	H	H
<b>6:</b>	H	CH <sub>3</sub>	OH	H	H
<b>7:</b>	H	CH <sub>3</sub>	OCH <sub>3</sub>	OH	CH <sub>3</sub>
<b>8:</b>	OH	H	H	H	CH <sub>3</sub>
<b>9:</b>	OH	CH <sub>3</sub>	OCH <sub>3</sub>	H	H
<b>10:</b>	OH	CH <sub>3</sub>	OCH <sub>3</sub>	OH	H
<b>11:</b>	OCH <sub>3</sub>	CH <sub>3</sub>	OCH <sub>3</sub>	H	H



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<b>12:</b>	H	H	H
<b>13:</b>	H	OH	CH <sub>3</sub>
<b>14:</b>	OCH <sub>3</sub>	H	H

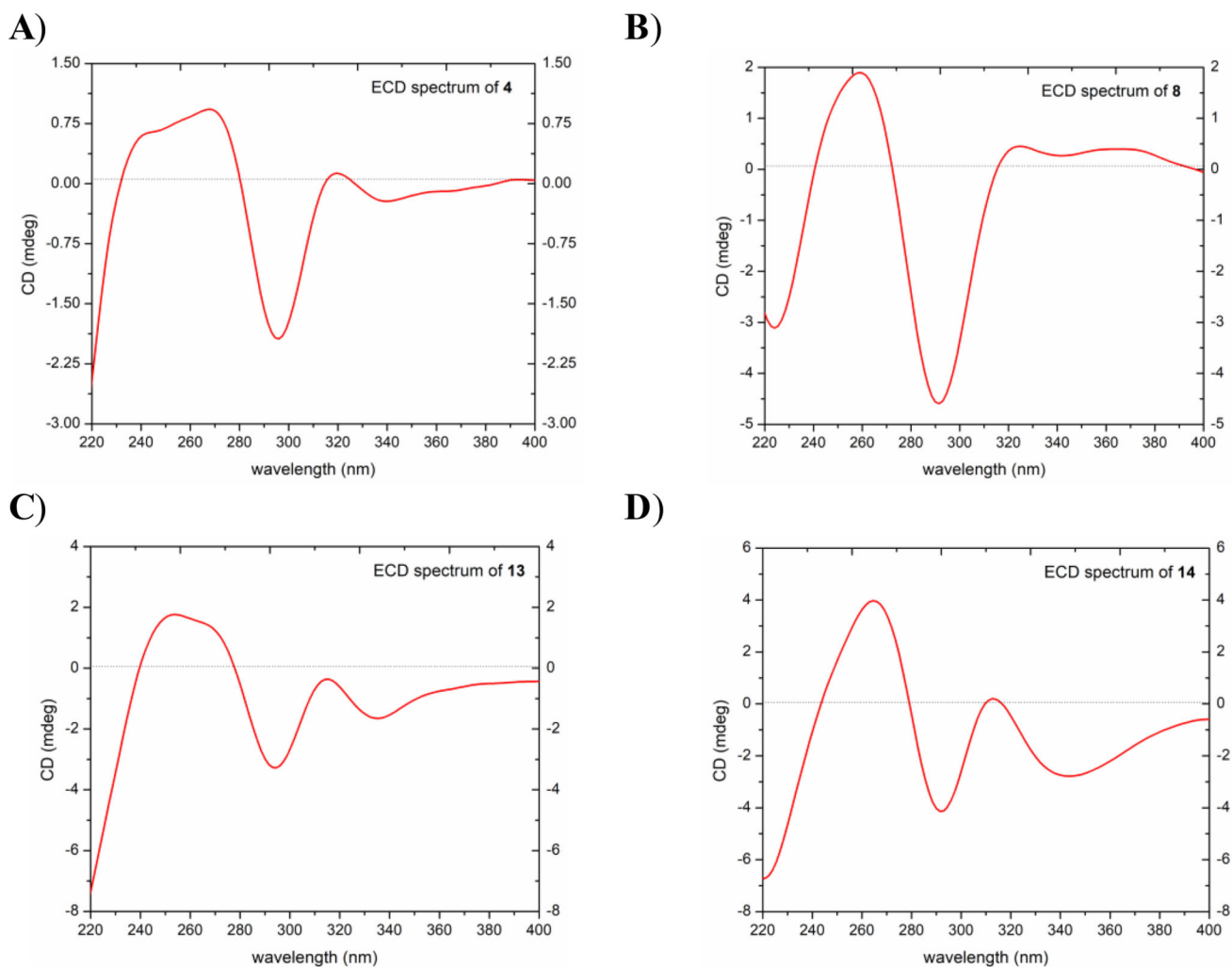
**15****16****17**

**Fig. 1.**  
Structures of compounds 1–17.

**4****8****13****14**

— COSY  
→ HMBC

**Fig. 2.**  
Key COSY and HMBC correlations of **4**, **8**, **13**, and **14**.



**Fig. 3.** ECD spectra for compounds A) **4**, B) **8**, C) **13**, and D) **14** [0.09 mM, MeOH, cell length 2 cm].

**Table 1**<sup>1</sup>H NMR Data for compounds **4**, **8**, **13**, and **14** (500 MHz in CDCl<sub>3</sub>)<sup>a</sup>.

position	<b>4</b>	<b>8</b>	<b>13</b>	<b>14</b>
2	4.12, dd (11.4, 7.1)	4.08, dd (11.4, 7.1)	4.06, d (11.2)	4.09, d (11.2)
	4.28, dd (11.4, 4.1)	4.25, dd (11.4, 4.1)	4.23, d (11.2)	4.36, d (11.2)
3	2.80, m	2.78, m		
6	6.06, d (2.3)		6.11, d (2.3)	6.16, s
8	5.97, d (2.3)	6.05, s	6.05, d (2.3)	
9	2.65, dd (13.9, 10.4)	2.69, dd (13.8, 10.6)	2.89, d (13.8)	2.94, d (14.1)
	3.12, dd (13.9, 4.5)	3.16, dd (13.8, 4.5)	2.93, d (13.8)	2.98, d (14.1)
10	3.81, s		3.85, s	3.91, s
11			3.88, s	3.83, s
12		3.91, s		
2'	6.75, d (1.3)	7.08, d (8.5)	6.82, d (2.1)	7.04, d (8.5)
3'		6.78, d (8.5)		6.75, d (8.5)
5'	6.81, d (7.9)		6.76, d (8.1)	
6'	6.66, dd (7.9, 1.3)		6.65, dd (8.1, 2.1)	
3-OH			3.36, s	
5-OH	12.11, s	11.78, s	11.25, s	11.21, s
6-OH		5.03, br. s		
7-OH		4.81, br. s		
3'-OH	5.20 or 5.33 <sup>b</sup> , br. s		5.58, s	
4'-OH	5.20 or 5.33 <sup>b</sup> , br. s			4.83, br. s

<sup>a</sup>  $\delta$  in ppm, mult (*J* in Hz);<sup>b</sup> Could be swapped



**Table 2**<sup>13</sup>C NMR Data for **4**, **8**, **14** (125 MHz), and for **13** (175 MHz) in CDCl<sub>3</sub>.

position	<b>4</b>	<b>8</b>	<b>13</b>	<b>14</b>
2	69.1	69.4	71.9	72.5
3	46.9	47.2	72.3	72.2
4	198.0	198.8	198.3	198.6
4a	102.7	102.5	100.6	100.4
5	164.6	148.2	164.1	160.0
6	95.1	156.1	95.5	93.7
7	168.0	127.4	168.8	162.3
8	94.0	91.1	94.6	129.9
8a	163.0	154.8	162.9	153.5
9	32.3	32.1	41.0	40.9
10	55.8		56.0	56.6
11			56.0	61.7
12		56.5		
1'	131.0	130.1	127.3	126.2
2'	116.3	130.5	116.8	131.9
3'	143.8	115.7	145.4	115.4
4'	142.4	154.5	145.9	155.0
5'	115.7		110.5	
6'	121.8		122.2	

**Table 4**Cytotoxic activities of compounds **2**, **4**, **7**, **13**, and **14**.

compound <sup>a</sup>	IC <sub>50</sub> values in $\mu\text{M}$ <sup>b</sup>		
	MDA-MB-435	Ovcar3	MDA-MB-231
<b>2</b>	1.6	14.2	9.5
<b>4</b>	14.3	17.5	24.8
<b>7</b>	2.0	3.6	10.8
<b>13</b>	14.4	>25	>25
<b>14</b>	15.2	>25	>25
<b>Taxol</b> <sup>c</sup>	0.0001	0.0015	0.17

<sup>a</sup>Compounds **1**, **3**, **5**, **6**, **8–12**, **15–17** were inactive, IC<sub>50</sub> values >25  $\mu\text{M}$ .

<sup>b</sup>IC<sub>50</sub> is the concentration to inhibit 50% of growth with a 72 h incubation.

<sup>c</sup>Positive control.