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-dihydropunctatin (**4**), 6-hydroxy-8-demethoxy-4′-*O*-methyl-3,9-dihydropunctatin (**8**), 7,4′-*O*-dimethyl-8-demethoxy-3,3′-dihydroxy-3,9-dihydropunctatin (**13**), and 7-*O*-methyl-3-hyroxy-3,9-dihydropunctatin (**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_{50} values of 1.6 and 2.0 μM, respectively, and were essentially equipotent against the OVCAR3 (ovarian) cancer cell line with IC_{50} values of 9.5 and 10.8 μM, respectively. However, compound **7**, with an IC_{50} value of 3.6 μM, was the most potent against the MDA-MB-231 (breast) cancer cell line.

Keywords: Homoisoflavonoids | *Bellevalia* | Bulbs | Absolute configuration | Cytotoxicity | Human cancer cell lines

Article:

1. Introduction

Homoisoflavonoids are a rare subclass of flavonoids possessing an extra carbon atom in their skeleton [1, 2, 3, 4, 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, 2, 3, 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^2, 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, 9, 10]. Within these different zones, thirteen vegetation types are identified, reflecting Jordan's diverse landscape, climate, and geology [8,11]. >2600 vascular plant species belonging to 113 family and 810 genera are reported to grow in the wild, of which 100 are endemic and >70 species are considered extinct [8]. Hence, studies of Jordan's wild plants have been initiated [12, 13, 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. eigii*) was investigated recently, yielding a set of homoisoflavoinids [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-dihydropunctatin (**4**), 6-hydroxy-8-demethoxy-4′-*O*-methyl-3,9-dihydropunctatin (**8**), 7,4′-*O*-dimethyl-8-demethoxy-3,3′-dihydroxy-3,9-dihydropunctatin (**13**), and 7-*O*-methyl-3-hyroxy-3,9-dihydropunctatin (**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 ^1H and 125 MHz for ^{13}C (JEOL Ltd.) or an Agilent 700 MHz NMR spectrometer (Agilent Technologies), equipped with a cryoprobe, operating at 700 MHz for ^1H and 175 MHz for ^{13}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 C_{18} analytical (5 μm; 250 × 4.6 mm) and preparative (5 μm; 250 × 21.2 mm) columns (both from Phenomenex) along with Atlantis T3 C_{18} analytical (5 μm;

250 × 4.6 mm) and semipreparative (5 μm; 250 × 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 $CHCl_3$ by soaking at rt. The solvent was evaporated under reduced pressure to yield the $CHCl_3$ extract (3.1 g), which was reconstituted in a 500 mL mixture of 5:4:1 H_2O:$CHCl_3$: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 MeOH:CH_3CN and 200 mL of hexanes and transferred into a separatory funnel. The biphasic solution was shaken vigorously. The MeOH/MeCN layer was evaporated to dryness under vacuum. The dried MeOH/CH_3CN layer (2.1 g) was dissolved in $CHCl_3$ and mixed with Celite 545. Normal-phase flash chromatography was run using a gradient solvent system of hexanes-$CHCl_3$-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 MeOH-H_2O (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 CH_3CN-H_2O (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 CH_3CN-H_2O (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 MeOH-H_2O (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 MeOH-H_2O (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 CH_3CN-H_2O (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 CH_3CN-H_2O (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 MeOH-H_2O (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 subtractions. Subfraction 1 (13.2 mg) was further purified using semipreparative (Atlantis T3 column) and a gradient solvent system of 30:70 to 40:60 CH_3CN-H_2O (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 semipreaprative HPLC method over Atlantis T3 column and an isocratic solvent system of 28:72 CH_3CN-H_2O (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_3CN-H_2O (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_3CN-H_2O (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 (*c* 0.1, MeOH)]; UV (MeOH) λ_{max} (log ε) 334 (3.23), 288 (3.98), 220 (3.94) nm; ECD (*c* 0.9×10^{-4} M, MeOH) λ ($\Delta\varepsilon$) 244 (+0.68) nm, 269 (+0.96) nm, 295 (−2.07), 320 (+0.18) nm (Fig. 3); HRESIMS *m*/*z* 317.1016 $[M + H]^+$ (calcd for $C_{17}H_{17}O_6$, 317.1020).

2.3.2. 6-Hydroxy-8-demethoxy-4′-*O*-methyl-3,9-dihydropunctatin (8)

Light yellow amorphous powder; $[\alpha]_D^{28}$ – 36 (*c* 0.1, MeOH)]; UV (MeOH) λ_{max} (log ε) 360 (3.32), 291 (4.05), 242 (3.94), 229 (3.99) nm; ECD (*c* 0.9×10^{-4} M, MeOH) λ ($\Delta\varepsilon$) 260 (+2.02) nm, 293 (−4.79), 323 (+0.50) nm (Fig. 3); HRESIMS *m*/*z* 317.1016 $[M + H]^+$ (calcd for $C_{17}H_{17}O_6$, 317.1020).

2.3.3. 7,4′-*O*-dimethyl-8-demethoxy-3,3′-dihydroxy-3,9-dihydropunctatin (13)

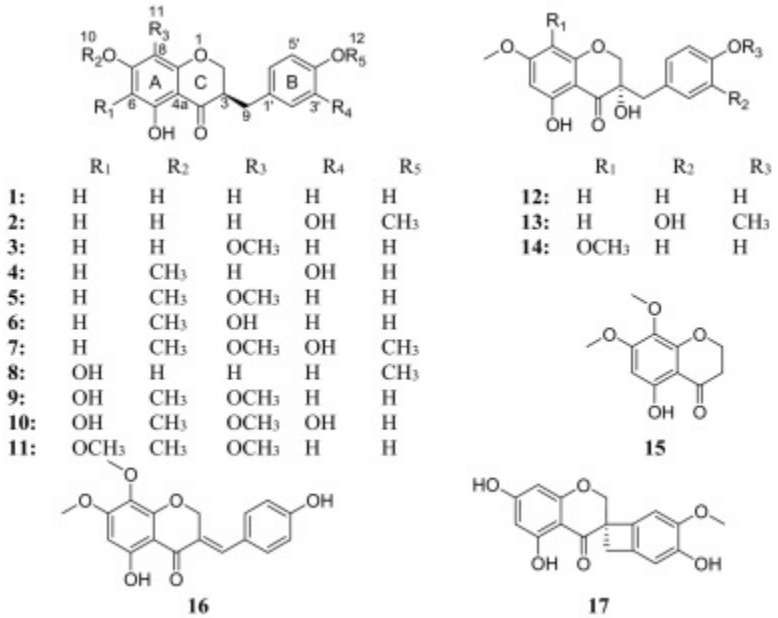
White amorphous powder; $[\alpha]_D^{28}$ – 96 (*c* 0.05, MeOH)]; UV (MeOH) λ_{max} (log ε) 334 (3.35), 288 (4.08), 218 (4.10) nm; ECD (*c* 0.9×10^{-4} M, MeOH) λ ($\Delta\varepsilon$) 253 (+1.80) nm, 295 (−3.52), 320 (−0.53) nm (Fig. 3); HRESIMS *m*/*z* 347.1122 $[M + H]^+$ (calcd for $C_{18}H_{19}O_7$, 347.1125).

2.3.4. 7-*O*-methyl-3-hyroxy-3,9-dihydropunctatin (14)

Light yellow amorphous powder; $[\alpha]_D^{28}$ – 170 (*c* 0.1, MeOH)]; UV (MeOH) λ_{max} (log ε) 348 (3.58), 292 (4.15), 224 (4.15) nm; ECD (*c* 0.9×10^{-4} M, MeOH) λ ($\Delta\varepsilon$) 265 (+4.16) nm, 292 (−4.53), 313 (+0.42) nm (Fig. 3); HRESIMS *m*/*z* 347.1124 $[M + H]^+$ (calcd for $C_{18}H_{19}O_7$, 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_2 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 5000 cells were seeded per well of a 96-well clear, flat-bottom plate (Microtest 96®, Falcon) and incubated overnight (37 °C in 5% CO_2). 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® AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, WI) that measured viable cells. IC_{50} values are expressed in µM relative to the solvent (DMSO) control. Taxol was used as a positive control.



	R_1	R_2	R_3	R_4	R_5
1:	H	H	H	H	H
2:	H	H	H	OH	CH_3
3:	H	H	OCH_3	H	H
4:	H	CH_3	H	OH	H
5:	H	CH_3	OCH_3	H	H
6:	H	CH_3	OH	H	H
7:	H	CH_3	OCH_3	OH	CH_3
8:	OH	H	H	H	CH_3
9:	OH	CH_3	OCH_3	H	H
10:	OH	CH_3	OCH_3	OH	H
11:	OCH_3	CH_3	OCH_3	H	H

	R_1	R_2	R_3
12:	H	H	H
13:	H	OH	CH_3
14:	OCH_3	H	H

15

16

17

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

3. Results and discussion

Air-dried bulbs of *B. flexuosa* were extracted with $CHCl_3$, and the resulting crude extract was reconstituted in a mixture of 5:4:1 H_2O:$CHCl_3$:MeOH. The dried organic extract was reconstituted in 1:1 MeOH/CH_3CN and then defatted using hexanes. The resulting dried MeOH/CH_3CN extract was fractionated using normal phase flash chromatography. HPLC methods, preparative and semipreprative, 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-trihydroxyhomoisoflavanone (**1**) [6], 3′-hydroxy-3,9-dihydroeucomin (**2**) [6], 3,9-dihydropunctatin (**3**) [20], 7-*O*-methyl-3,9-dihydropunctatin (**5**) [6], 8-*O*-demethyl-7-*O*-methyl-3,9-dihydropunctatin (**6**) [6], 7,4′-di-*O*-methyl-3′-hydroxy-3,9-dihydropunctatin (**7**) [6], 6-hydroxy-7-*O*-methyl-3,9-dihydropunctatin (**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-dihydropunctatin (**12**) [6], 5-hydroxy-7,8-dimethoxychroman-4-one (**15**) [6], 7-*O*-methylpunctatin (16) [6], and isomuscomosin (**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 $C_{17}H_{16}O_6$ (10 degrees of unsaturation) as determined by HRESIMS (*m*/*z* 317.1016 $[M + H]^+$, calcd. 317.1020), which was further supported by ^1H, ^{13}C, and edited HSQC NMR data (Table 1, Table 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_3–7′ (δ_H 3.88, s) to C-4′ (δ_C 145.5) in **2** and from H_3–10 (δ_H 3.81, s) to C-7 (δ_C 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_H 5.97, d, J = 2.3) to C-4a (δ_C 102.7), C-6 (δ_C 95.1), C-7 (δ_C 168.0), and C-8a (δ_C 163.0); from H-6 (δ_H 6.06, d, J = 2.3) to C-5 (δ_C 164.6), C-7, C-8 (δ_C 94.0), and C-4a, from 5-OH (δ_H 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_H 6.75, d, J = 1.3) to C-9 (δ_C 32.3), C-4′ (δ_C 142.4), and C-6′ (δ_C121.8); from H-5′ (δ_H 6.81, d, J = 7.9) to C-1′ (δ_C 131.0) and C-3′ (δ_C 143.8); from H-6′ (δ_H6.66, dd, J = 7.9, 1.3) to C-9, C-2′ (δ_C 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 3*R* configuration [22]. Compound **4** showed a negative Cotton effect at 295 nm ($\Delta\varepsilon = -2.07$) in the ECD spectrum, supporting an *R*-configuration at C-3 (Fig. 3).

Table 1. ^{1}H NMR data for compounds **4**, **8**, **13**, and **14** (500 MHz in $CDCl_3$)[a].

position	4	8	13	14
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[b], br. s		5.58, s	
4'-OH	5.20 or 5.33[b], br. s			4.83, br. s

[a]δ in ppm, mult (J in Hz); [b]Could be swapped.

Table 2. ^{13}C NMR data for **4**, **8**, **14** (125 MHz), and for **13** (175 MHz) in $CDCl_3$.

position	4	8	13	14
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	

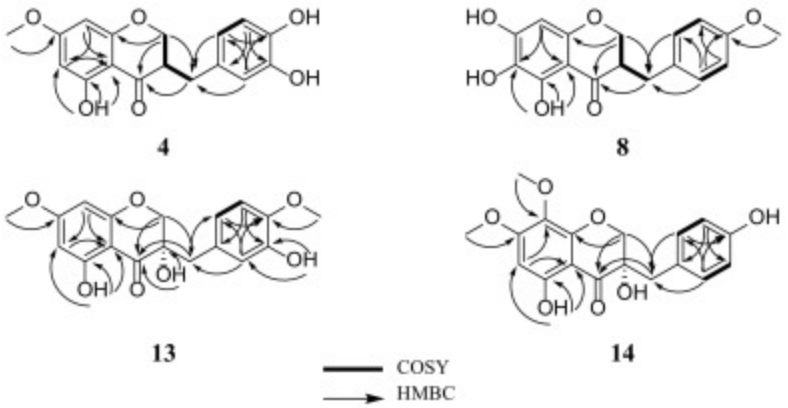


Fig. 2. Key COSY and HMBC correlations of **4**, **8**, **13**, and **14**.

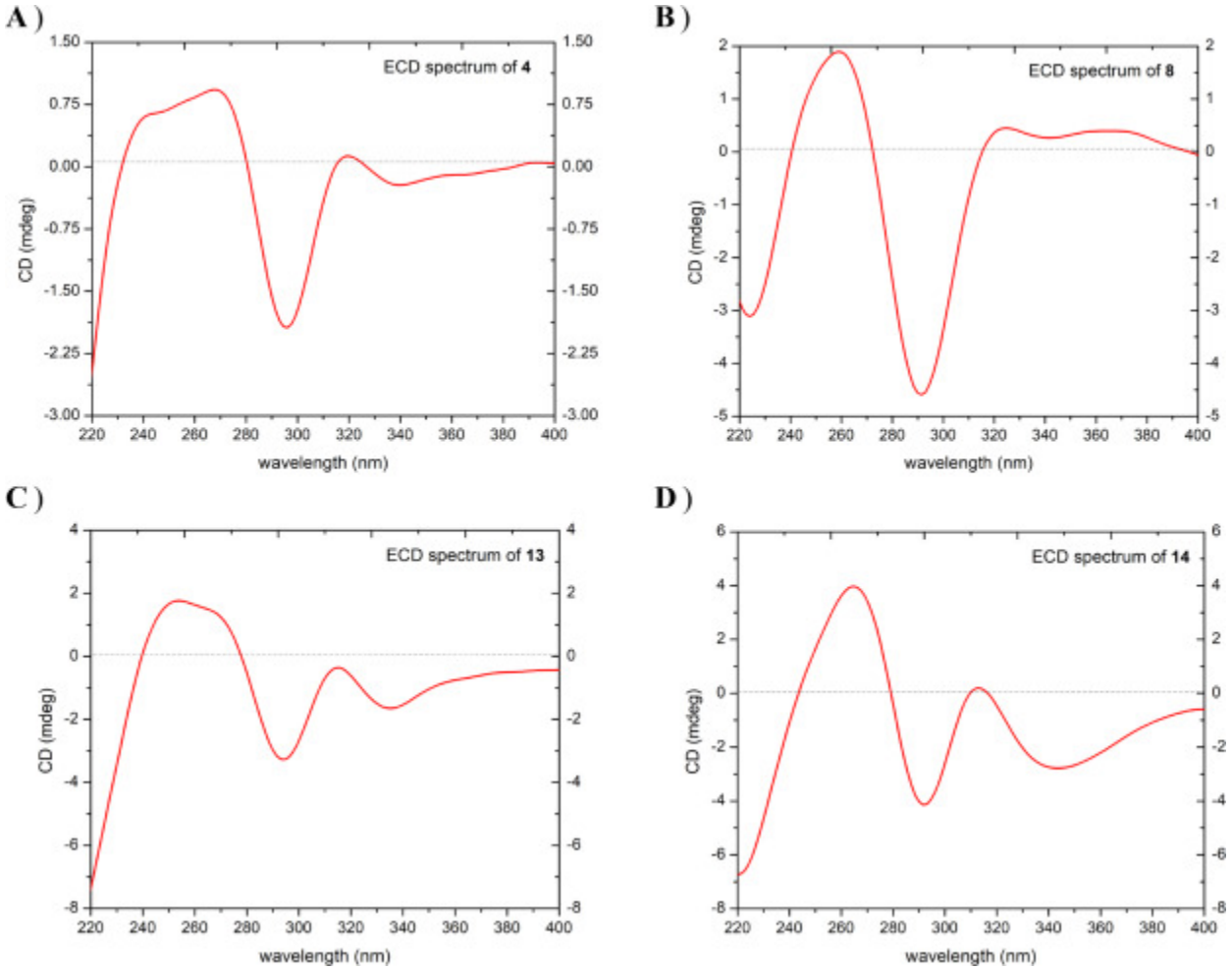


Fig. 3. ECD spectra for compounds A) **4**, B) **8**, C) **13**, and D) **14** [0.09 mM, MeOH, cell length 2 cm].

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) (Table 1, Table 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 (δ_H/δ_C 3.95/61.7 for H_3–11/C-11) in **3** was replaced by an aromatic proton singlet (δ_H/δ_C 6.05/91.1 for H-8/C-8) in **8**. In addition, the aromatic proton singlet (δ_H/δ_C 6.13/96.0 for H-6/C-6) in **3** was replaced by an exchangeable proton singlet (δ_H 5.03 for 6-OH) in **8**. Moreover, the exchangeable proton singlet in **3** (δ_H 4.75 for 4′-OH) was replaced by a methyl group (δ_H/δ_C 3.91/56.5 for H_3–7′/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_3–7′ (δ_H 3.91, s) to C-4′ (δ_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**($\Delta\varepsilon = -4.79$) 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 $C_{18}H_{18}O_7$(Table 1, Table 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 (δ_H/δ_C 3.88/56.0) and a hydroxyl group at δ_H 5.58 (3'-OH) consistent with the 30 amu difference in the HRMS data of **13** relative to **12**. The aromatic A_2B_2 system of ring B in **12** was replaced by an ABM spin system in **13** (δ_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_3 protons to C-4′ (δ_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 ($\Delta\varepsilon = -3.52$) 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 $C_{18}H_{18}O_7$ (Table 1, Table 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 ^1H and ^{13}C NMR data (δ_H/δ_C 3.83/61.7), consistent with the 30 amu difference in the HRMS data of **14** relative to **12**, replacing the aromatic doublet (δ_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-hyroxy-3,9-dihydropunctatin. A negative cotton effect ($\Delta\varepsilon = -4.53$) 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 IC_{50} values of 1.6, 14.2, 9.5 and 2.0, 3.6, and 10.8 μM, respectively (Table 3). Compound **2** was reported to be active against colon cancer (HT-29 cell line ED_{50} = 2.78 μM) and breast cancer (MDA-MB-435 cell line ED_{50} = 1.33 μM). Compounds **2** and **7** were reported previously by our group to be active against breast cancer (MDA-MB-435 cell line) with IC_{50} values of 1.0 and 1.1 μM, respectively [6]. Moreover, compound **7** showed moderate activity when tested against colon cancer (HT-29 cell line IC_{50} = 17.3 μM) [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 IC_{50} values of 14.3, 17.5, and 24.8 μM 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 IC_{50} values of 1.6 and 9.5 μM, respectively. Similarly, although compound **10** was inactive, compound **7**, with a 4′-methoxy substituent, showed IC_{50} values of 2.0, 3.6, and 10.8 μM 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 IC_{50} value of 14.4 μM. 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 IC_{50} value of 15.2 μM against the MDA-MB-435 cancer cell line.

Table 3. Cytotoxic activities of compounds **2**, **4**, **7**, **13**, and **14**.

compound[a]	**IC_{50} values in μM**[b]		
	MDA-MB-435	**Ovcar3**	**MDA-MB-231**
2	1.6	14.2	9.5
4	14.3	17.5	24.8
7	2.0	3.6	10.8
13	14.4	>25	>25
14	15.2	>25	>25
Taxol[c]	0.0001	0.0015	0.17

[a]Compounds **1, 3, 5, 6, 8–12, 15–17** were inactive, IC_{50} values >25 μM. [b]IC_{50} is the concentration to inhibit 50% of growth with a 72 h incubation. [c]Positive control.

Conflict of interest

All the authors have no conflict of interest.

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