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Decorosides A and B, Cytotoxic Flavonoid Glycosides from the Leaves of *Rhododendron decorum*

Mostafa E. Rateb[a,b,*], Hossam M. Hassan[b], El-Shaimaa A. Arafa[c], Marcel Jaspars[a] and Rainer Ebel[a]

[a]*Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Meston Walk, Aberdeen AB24 3UE, Scotland, U.K.*

[b]*Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62511, Egypt*

[c]*Department of Pharmacology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62511, Egypt*

ratebnplab@gmail.com

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Bioassay and NMR-guided fractionation of the methanolic extract of *Rhododendron decorum* leaves resulted in the isolation of two new flavonoid glycosides, 5,7-dihydroxy-6,8-dimethyldihydroflavanone-7-*O*-α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside (decoroside A, **1**) and its 3-hydroxy congener (decoroside B, **2**), along with five known compounds myricitrin (**3**), afzelin (**4**), (–)-epicatechin (**5**), (+)-catechin (**6**), and ampeloptin (**7**). The structures of the isolated compounds were elucidated by extensive interpretation of their spectral data. Biological evaluation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed promising cytotoxic activities of these compounds against different cancer cell lines.

Keywords: *Rhododendron decorum*, Decoroside, Flavonoid, Cytotoxic activities.

Natural products are the most reliable source of new anticancer entities. Around 63% of anticancer drugs introduced over the last 25 years are either natural products or inspired by natural products. Natural products represent the most important sources for drug discovery. Higher plants are considered one of the foremost sources of therapeutic agents for mankind, and of about 270,000 terrestrial plants that have been taxonomically classified, *ca*. 10,000 are used for medicinal purposes [1]. Moreover, approximately 200,000 secondary metabolites of plant origin have been reported to date [2]. However, as the rate of re-isolation of plant secondary metabolites has significantly increased in the last two decades, the use of advanced spectral techniques and database dereplication has had a great impact by reducing re-isolation and simplifying the finding of new metabolites in plant extracts for further purification.

Since the weather conditions have a great impact on secondary metabolite profile [3, 4], we decided to screen some extracts from northern Scottish plants for their cytotoxicity and dereplicated the active hits for new secondary metabolites using NMR spectroscopy. During this process, we came across an extract with high activity from a species of the genus *Rhododendron*. The genus *Rhododendron*, which contains about 850 species, is one of the main genera in the family Ericaceae, distributed in the Northern Hemisphere, mainly in Asia. For decades, the dried stems, leaves, and flowers of some *Rhododendron* species have been used in traditional medicine to treat diseases such as asthma, and also as an expectorant, analgesic and anti-inflammatory [5]. Screening of this genus for secondary metabolites started at late 1960s, and, in particular, during the last 20 years the phytochemical investigation of *Rhododendron* has resulted in the isolation and characterization of more than 200 secondary metabolites of different chemical classes, mainly flavonoids and diterpenoids [5]. *R. decorum* is a woody perennial tree, grown as an ornamental plant. Previous investigation of this species has led to the isolation of different secondary metabolites, especially flavonoids of diverse subclasses as well as diterpenoids of the grayanane type [6-8]. So far, there are few reports regarding the cytotoxic activity of the secondary metabolites isolated from this genus against different cancer cell lines [5]. Therefore, the ultimate objective of this study was to isolate and identify the bioactive components of this extract and assess their cytotoxic activity against different cell lines.

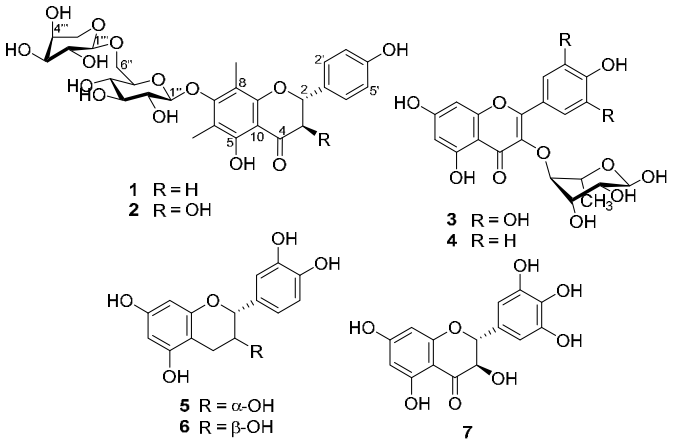

Figure 1: Structures of compounds **1**-**7** from the leaves of Rhododendron decorum.

The methanolic extract of *R. decorum* leaves was selected based on cytotoxicity-guided screening. Application of different chromatographic techniques resulted in the isolation of two new flavonoid glycosides, farrerol-7-*O*-α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside (decoroside A, **1**), and its 3-hydroxy congener (decoroside B, **2**), along with five known compounds, which, based on NMR spectroscopic interpretation, as well as comparison of their spectral characteristics with previously reported data, were identified as myricitrin (**3**) [9], afzelin (**4**) [10], (–)-epicatechin (**5**) [11], (+)-catechin (**6**) [11], and ampeloptin (**7**) [11] (Figure 1).

Table 1: ^{1}H (600 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for decorosides A (**1**) and B (**2**) in DMSO-d_6 (298 K).

	Decoroside A (**1**)		Decoroside B (**2**)	
No.	δ_C, mult.	δ_H, mult. (*J* in Hz)	δ_C, mult.	δ_H, mult. (*J* in Hz)
2	78.3, CH	5.47, dd (13.1, 2.6)	72.0, CH	5.08, d (11.7)
3	42.4, CH_2	3.36, m	82.9, CH	4.67, d (11.7)
		2.75, dd (15.3, 2.7)		
4	198.6, C		200.3, C	
5	157.9, C		157.7, C	
6	110.9, C		111.1, C	
7	161.0, C		161.2, C	
8	110.0, C		110.2, C	
9	157.4, C		157.1, C	
10	104.8, C		103.6, C	
1′	129.1, C		127.8, C	
2′/6′	128.2, CH	7.36, d (8.2)	129.5, CH	7.36, d (8.3)
3′/5′	115.2, CH	6.80, d (8.2)	115.0, CH	6.79, d (8.3)
4′	157.6, C		157.7, C	
6-Me	8.7, CH_3	2.07, s	8.7, CH_3	2.09, s
8-Me	9.3, CH_3	2.03, s	9.2, CH_3	1.98, s
5-OH		12.13, s		11.91, s
2″-OH		5.57, d (5.1)		
4″-OH		5.07, d (4.9)		
5″-OH		4.97, br s		
2‴-OH		6.69, d (4.8)		
1″	103.8, CH	4.62, d (7.6)	103.9, CH	4.61, d (7.6)
2″	74.0, CH	3.30, m	74.0, CH	3.30, t (8.7)
3″	69.5, CH	3.22, t (8.1)[a]	69.5, CH	3.23, t (8.3)[a]
4″	69.7, CH	3.15, m	69.7, CH	3.15, t (8.9)
5″	76.3, CH	3.24, m	76.3, CH	3.25, t (8.5)[a]
6″	68.1, CH_2	3.82, d (11.6)	68.1, CH_2	3.83, d (11.5)
		3.54, dd (11.8, 5.6)		3.54, dd (12.0, 6.2)
1‴	103.4, CH	4.03, d (6.7)	103.4, CH	4.04, d (6.7)
2‴	73.2, CH	2.88, m	73.2, CH	2.87, t (7.9)
3‴	76.3, CH	2.93, t (7.8)[a]	76.5, CH	2.94, t (8.1)[a]
4‴	69.5, CH	3.24, m	69.5, CH	3.24, m
5‴	65.4, CH_2	3.64, dd (11.3, 5.2)	65.5, CH_2	3.65, dd (11.3, 5.3)
		2.91, m		2.92, m

[a] Extracted from 1D-TOCSY spectra.

Compound **1** was isolated as optically active yellow powder. HRESIMS analysis established its molecular formula as $C_{28}H_{34}O_{15}$. It showed IR (3417 and 1635 cm^{-1}) and UV absorptions (229 and 285 nm) indicative of a flavanone nucleus. The ^{1}H NMR spectrum of **1** (Table 1) showed resonances accounting for two aromatic methyl groups at δ_H 2.07 (3H, s) and δ_H 2.03 (3H, s), a 1,4-disubstituted aromatic system [δ_H 6.80 (2H, d, *J* = 8.2 Hz) and δ_H 7.36 (2H, d, *J* = 8.2 Hz)], and an ABX-system [δ_H 3.36 (1H, m), 2.75 (1H, dd, *J* = 15.3, 2.7 Hz)], and 5.47(1H, dd, *J* = 13.1, 2.6 Hz)] corresponding to a C-ring of a flavanone. These data coincide with that of farrerol aglycone [11]. The ^{13}C NMR spectrum displayed 28 carbon resonances (Table 1), of which 17 were virtually identical to those reported for farrerol [11], while the remaining 11 resonances (δ_H 3.0-5.0) were indicative of the presence of a diglycoside with one hexose and one pentose moiety. ^{1}H and ^{13}C NMR data (Table 1), together with assessment of the coupling constants, in addition to gCOSY (Figure 2) and 1D TOCSY analyses suggested that the sugar moiety of **1** was identical to that of miconioside A, i.e. α-L-arabinopyranosyl-(1→6)-β-D-glucopyranose [12,13]. The two sugar moieties were further confirmed by acid hydrolysis of **1** upon which β-D-glucopyranose and α-L-arabinopyranose were detected by co-chromatography on TLC in comparison with authentic sugar samples. Analysis of the HMBC spectrum (Figure 2) revealed a ^{3}J correlation of H_2-6″ (δ_H 3.52, 3.84) of glucose with C-1‴ (δ_C 103.4) of arabinose, which confirmed the linkage between the two sugar moieties as α-L-arabinopyranosyl-(1→6)-β-D-glucopyranose. The correlation of H-1″ (δ_H 4.62) to C-7 (δ_C 161.0) revealed the attachment of the sugar moiety to position 7 of the aglycone. The relative configuration of 1 was assigned based on the NOESY correlations (Figure 2). Comparing the optical rotation of the aglycone with the previously published data [12] indicated that the

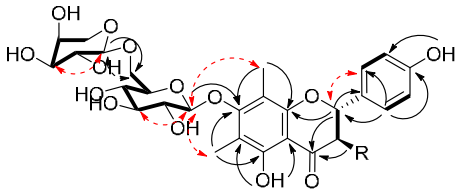

Figure 2: Selected COSY (▬), HMBC (⌒) and NOESY (⌒) correlations of decorosides.

absolute configuration at C-2 of the aglycone was *S*. On the basis of these data, **1** was identified as (2*S*)-farrerol-7-*O*-α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside, representing a new secondary metabolite for which we propose the name decoroside A.

HRESIMS analysis of compound **2** established its molecular formula as $C_{28}H_{34}O_{14}$, which was 16 mass units higher than **1,** indicating the presence of an additional oxygen atom in the form of a hydroxyl substituent. The ^{1}H and ^{13}C NMR data (Table 1) showed that the CH_2 signal at position 3 in **1** was replaced by an oxygenated methine function (δ_H/δ_C 4.67/72.0) in **2**, while the coupling constant observed for H-3 (d, *J* = 11.7) indicating that the hydroxyl function was β-oriented. All remaining ^{1}H and ^{13}C NMR resonances were almost identical to those of **1**. The absolute stereochemistry was assigned as 2*R*,3*R*, consistent with the previous discussion of compound **1**, the coupling constant of H-3, as well as the previously published data of the same aglycone [14]. Based on this evidence, compound **2** was identified as 6,8-dimethyldihydrokaempferol-7-*O*-α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside, representing a new natural product for which the name decoroside B is suggested.

Cytotoxic activity: All the isolated phenolic compounds were evaluated *in vitro* for their cytotoxic activity against four cancer cell lines, i.e. the human breast cancer cell line (MCF-7), human hepatoma cell line (Hep3B), human colon adenocarcinoma grade II cell line (HT29), and human alveolar basal epithelial cell line (A549) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. According to Table 2, almost all compounds showed moderate to strong cytotoxicity against the four cell lines. Compound **3** showed a potent cytotoxic activity against the MCF-7 cell line, followed by **1**, **2** and **4** (Table 2). Compound **4** showed moderate cytotoxicity against all cell lines, except HT29, where it exhibited strong activity. On the other hand, compounds **1** and **2** showed strong cytotoxicity against both MCF-7 and A549, while compounds **5** and **6** showed either weak or no activity against MCF-7, Hep3B, and A549 cell lines (Table 2). Based on these results, we could conclude that glycosylation plays a role in cytotoxic activity, while losing the glycosidic linkage diminishes the cytotoxic activities, especially against MCF-7 and Hep3B, which may play an important role in solubilization inside the cell line. On the other hand, loss of C=O, as in compounds **5** and **6**, led to the loss of cytotoxic effect.

Bioactivity and NMR-guided isolation still play the major role in the discovery of new natural products. In this study, cytotoxicity-guided isolation using different chromatographic techniques has led to the isolation of two new flavonoid glycosides, decorosides A-B, in addition to five previously isolated phenolic compounds. Weather conditions could have a great impact on secondary metabolite profiles as these two new metabolites were not traced before in the genus *Rhododendron*. The screened compounds showed moderate to strong cytotoxic activity against a panel of cancer cell lines.

Table 2: *In vitro* cytotoxicity of compounds **1-7** against four cell lines.

Compds	IC_{50} (μM) [a]			
	MCF-7	Hep3B	HT29	A549
1	10.3 ± 1.7	51.9 ± 3.9	37.6 ± 2.1	11.1 ± 2.1
2	12.1 ± 2.9	48.4 ± 3.0	36.9 ± 1.8	10.5 ± 1.7
3	6.2 ± 1.1	14.2 ± 1.2	42.6 ± 2.2	>100
4	14.9 ± 1.9	17.7 ± 2.0	10.9 ± 1.7	18.2 ± 2.5
5	89.5 ± 4.2	>100	82.1 ± 4.6	86.7 ± 2.4
6	>100	>100	73.5 ± 2.7	92.6 ± 3.0
7	>100	18.6 ± 2.3	12.2 ± 1.7	15.9 ± 2.1
Doxorubicin	1.4 ± 0.02	2.4 ± 0.07	0.4 ± 0.01	1.9 ± 0.02

[a] Values are mean ± S.D. of three independent experiments.

Experimental

General: Optical rotations were recorded using a Perkin-Elmer 343 polarimeter. UV and IR spectra were measured on a Perkin-Elmer Lambda 25 UV/vis spectrometer and a Thermo Nicolet IR 100 FT/IR spectrometer, respectively. ^{1}H, ^{13}C, and 2D NMR experiments were acquired on a Varian VNMRS 600 MHz spectrometer. High resolution mass spectral data were obtained from a Thermo Instruments MS system (LTQ XL/ LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump). The following conditions were used: capillary voltage 45 V, capillary temperature 320°C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100–2000 amu (maximum resolution 30000). HPLC separations were made using a Phenomenex reversed-phase (C_{18}, 250 × 10 mm, L × i.d.) column connected to an Agilent 1200 series binary pump and monitored using an Agilent photodiode array detector. Detection was carried out at 220, 230, 254, 280, and 350 nm.

Plant material: Fresh leaves of *R. decorum* L., family Ericaceae, were collected from the Cruickshank Botanic Garden, University of Aberdeen in March 2011, dried in shade and powdered. A voucher specimen is on deposit at the Herbarium of The Cruickshank Botanic Garden, University of Aberdeen (voucher #CBG 2009-0029).

Extraction and isolation: The dried powdered plant material (270 g) was extracted by percolation with 95% EtOH (800 mL × 3). The extracts were combined and concentrated *in vacuo* to dryness (6.3 g). The residue was suspended in 250 mL H_2O and fractionated with *n*-hexane (3 × 250 mL), CH_2Cl_2 (4 × 250 mL) and EtOAc (4 × 250 mL). Quick NMR analysis revealed that the EtOAc fraction had the most diverse metabolic profile. This fraction (180 mg) was loaded onto a Sephadex LH-20 column equilibrated with MeOH and 2 fractions were collected. The first (70 mg) was further purified by reversed-phase HPLC using a gradient of MeOH in H_2O as eluent (10 – 70% over 50 min.) at a flow rate of 1.50 mL min^{-1} and afforded the pure compounds **1** (6 mg), **2** (4.5 mg), **3** (3 mg), and **4** (1.5 mg). The second fraction (100 mg), also purified by the same procedure, afforded the pure compounds **5** (17 mg), **6** (3 mg), and **7** (11 mg).

Cytotoxicity assay

Cell culture: Human breast cancer cell line (MCF-7), human hepatoma cell line (Hep3B), human colon adenocarcinoma grade II cell line (HT29), and human alveolar basal epithelial cell line (A549) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium/F12 medium (DMEM/F-12, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco). All cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

Cell viability assay: The effect of the isolated compounds on cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in 6 replicates, as reported before [15, 16]. Briefly, MCF-7, Hep3B, HT29 and A549 cells were seeded in 96-well plates for 24 h, and treated with test agents in 5% FBS-supplemented DMEM/F-12 for 72 h. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. Doxorubicin was used as a positive control. After treatment, cells were incubated in the same medium containing 0.5 mg/mL MTT at 37°C for 2h. Reduced MTT was solubilised in DMSO (200 μL/well) for determination of absorbance at 570 nm using a microplate reader.

Decoroside A (1)
Yellow powder.
$[\alpha]^{20}_{D}$: –22.5 (*c* 0.1, CH_3OH).
IR (KBr): 3417, 1635, 1517, 1346 cm^{-1}.
UV/Vis (MeOH) λ_{max} ($\log\epsilon$): 229 (3.74), 285 (2.38), 358 (2.40) nm.
^{1}H and ^{13}C NMR: Table 1.
HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{28}H_{35}O_{15}$: 611.1970; found: 611.1964.

Decoroside B (2)
Yellow powder.
$[\alpha]^{20}_{D}$: –33.7 (*c* 0.1, CH_3OH).
IR (KBr): 3413, 1631, 1519, 1342 cm^{-1}.
UV/Vis (MeOH) λ_{max} ($\log\epsilon$): 229 (3.74), 285 (2.38), 360 (2.50) nm.
^{1}H and ^{13}C NMR: Table 1.
HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{28}H_{35}O_{14}$: 595.2021; found: 595.2029.

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Supporting Information Available: NMR spectra of **1-2** including ^{1}H, ^{13}C, COSY, HSQC, HMBC and NOESY in DMSO-d_6, are available as supporting information.

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