Spectroscopic Characterization and Antiproliferative Activity on HepG2 Human Hepatoblastoma Cells of Flavonoid *C*-Glycosides from *Petrorhagia velutina*

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Eight flavonoid *C*-glycosides, including three new analogues, have been isolated from leaf and root methanolic extracts of *Petrorhagia velutina*, a Mediterranean herbaceous plant. The antiproliferative activity against human hepatoblastoma cancer cell line HepG2 has been analyzed by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) test. Isoorientin (**4**) significantly reduces the proliferation of HepG2 cells as determined by the complete conversion of the tetrazolium probe into formazan after 48 h of exposure.

Flavonoids are naturally occurring polyphenolic metabolites present in plant organisms. Many studies correlate the beneficial health effects of the consumption of fruit and vegetables to the abundance of this class of compounds.[1] Additionally, flavonoids have been isolated from and/or identified in several medicinal plants and herbal remedies used in folk medicines around the world.[2]

These metabolites show a wide spectrum of biological activities,[3] e.g., antioxidant, antiallergic, anti-inflammatory, and antimutagenic, and some representative flavonoids, such as quercetin, catechin, gallocatechin, and apigenin, have been investigated as possible chemopreventive or chemoterapic agents against cancer.[4,5]

In the search for new bioactive secondary metabolites from natural sources, we reported the isolation and characterization of new antioxidant and antiproliferative phytochemicals from Mediterranean plants.[6,7] Recently, we undertook the phytochemical study of *Petrorhagia velutina*, a Eurimediterranean herb, belonging to the Caryophyllaceae family, typical of woodland and *macchia* vegetation.[8,9] In the present paper, the characterization of eight flavonoid *C*-glycosides, including three new analogues, and the evaluation of their antiproliferative activity against human hepatoblastoma cancer cell line HepG2 are reported.

Results and Discussion

The dried MeOH extract of *P. velutina* was submitted to multiple chromatographic steps, yielding eight flavonoid *C*-glycosides (Figure 1). The ^{1}H NMR spectrum of compound **1** (Table 1) showed five protons in the aromatic region: an AA′BB′ spin system as two aromatic doublets at δ_H 7.32 and 6.77 ($J = 8.4$ Hz) each integrating for two protons, and a one proton singlet at δ_H 6.31. The remaining part of the spectrum showed a doublet of doublets at δ_H 5.50, two doublets at δ_H 4.68 and 4.35 each integrating for one proton, a broadened one-proton doublet at δ_H 2.85, and further overlapped protons ranging from δ_H 3.75 to 2.80. ^{13}C NMR and HSQC experiments confirmed the presence of 26 carbons, identified as three methylene, 15 methine (two including the homotopic carbon pairs of the AA′BB′ system), a carbonyl carbon (δ_C197.8), and seven quaternary carbons.

COSY/TOCSY experiments (Table 1) showed correlations between the C-ring proton resonating at δ_H 5.50 (H-2) and the proton resonances at δ_H 2.85 and 3.19 (H-3b and H-3a), as well as cross-peaks between the anomeric proton doublet at δ_H 4.68 (H1-Glc),and a proton signal at δ_H 3.35 (H2-Glc) and of the doublet at

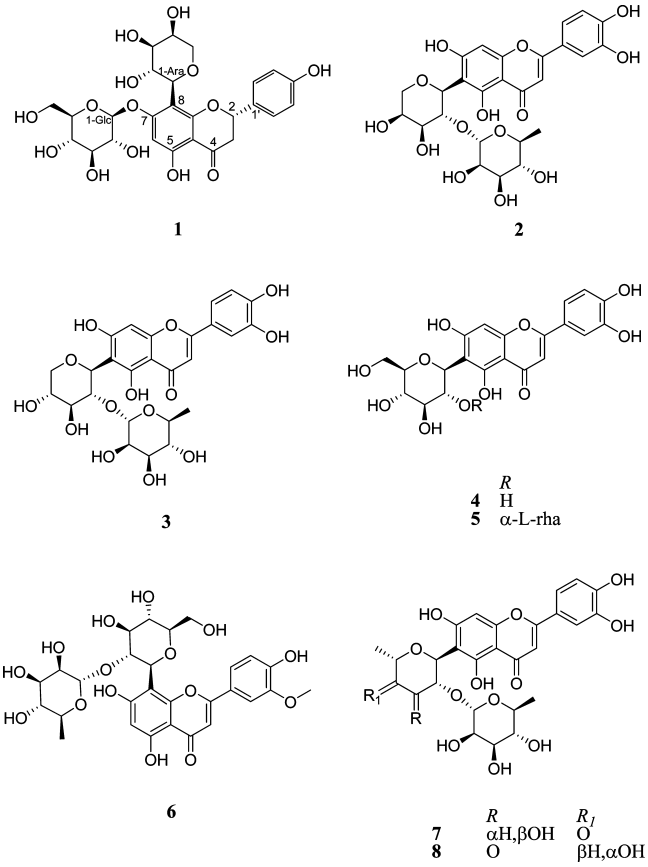

Figure 1. Flavonoid *C*-glycosides from *P. velutina*.

δ_H 4.35 (H1-Ara) with the signal at δ_H 4.31 (H2-Ara). The protons at δ_H 2.85 (H-3b), and 3.19 (H-3a) both correlated, in the HSQC experiment, to the carbon at δ_C 42.1 (C-3) and showed cross-peaks, in the HMBC spectrum, with the carbonyl carbon at δ_C 197.8 (C-4). The carbonyl carbon (C-4) furthermore showed long-range interactions with the doublet of doublets at δ_H 5.50 (H-2) and the aromatic proton at δ_H 6.31 (H-6). These data suggested compound **1** to be a flavanone with a *para*-substitued B ring and two glycosyl substituents.

On the basis of molecular mass and structural information obtained by ESIMS and ^{1}H and ^{13}C NMR experiments, it was possible to attribute a molecular formula, $C_{26}H_{30}O_{14}$, to compound **1**.

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Table 1. 1D and 2D NMR Data (300 MHz, DMSO-d_6) of Compound **1**

position	δ_C, mult	δ_H (J in Hz)	COSY (H→H)	HMBC[a] (H→C)	NOESY (H→H)
2	78.2, CH	5.50, dd (11.4, 2.7)	3a, 3b	1′, 2′, 6′, 3, 4, 9, 10	2′, 6′
3a	42.1, CH_2	3.19, overlap	2	1′, 2, 4	
3b		2.85, d (15.0)	2	1′, 2, 4	
4	197.8, C				
5	162.9, C				
6	96.4, CH	6.31, s		4, 5, 7, 8, 10	6-Glc
7	165.4, C				
8	107.7, C				
9	160.2, C				
10	103.1, C				
1′	128.7, C				
2′	127.9, CH	7.32, d (8.4)	3′	1′, 2, 3′, 4′, 6′	2, 3′
3′	115.2, CH	6.77, d (8.4)	2′	1′, 2′, 4′, 5′	2′
4′	157.5, C				
5′	115.2, CH	6.77, d (8.4)	6′	1′, 3′, 4′, 6′	6′
6′	127.9, CH	7.32, d (8.4)	5′	1′, 2, 2′, 4′, 5′	2, 5′
1-Glc	102.1, CH	4.68, d (7.5)	2-Glc	7, 3-Glc, 6-Glc	
2-Glc	73.3, CH	3.35, overlap	1-Glc, 3-Glc	3-Glc	
3-Glc	75.0, CH	3.33, overlap	2-Glc, 4-Glc	2-Glc	
4-Glc	69.8, CH	3.14, m	3-Glc, 5-Glc	5-Glc	
5-Glc	77.6, CH	3.26, overlap	4-Glc, 6a-Glc, 6b-Glc	4-Glc	
6a-Glc	60.2, CH_2	3.70, overlap	5-Glc, 6b-Glc	5-Glc	
6b-Glc		3.45, dd (12.0, 6.0)	5-Glc, 6a-Glc	5-Glc	
1-Ara	74.0, CH	4.35, d (9.9)	2-Ara	7, 8, 9, 5-Ara	
2-Ara	68.0, CH	4.31, m	1-Ara, 3-Ara	1-Ara	
3-Ara	74.9, CH	3.26, overlap	2-Ara, 4-Ara	2-Ara	
4-Ara	69.0, CH	3.69, overlap	3-Ara, 5a-Ara, 5b-Ara	5-Ara	
5a-Ara	70.2, CH_2	3.62, d (11.4)	4-Ara, 5b-Ara,	4-Ara	
5b-Ara		3.39, overlap	4-Ara, 5a-Ara	4-Ara	

[a] HMBC correlations, optimized for for 8 Hz, are from proton(s) stated to the indicated carbon. s, singlet; d, doublet; dd, double doublet; m, multiplet; overlap, overlapped.

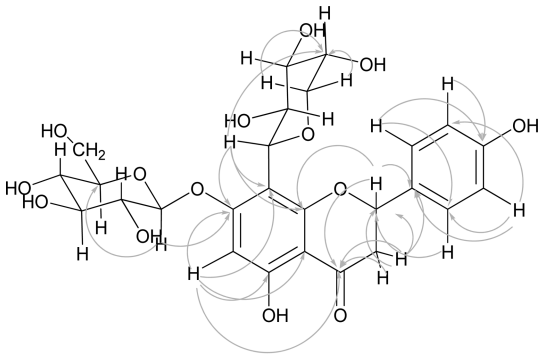
Table 2. TQ Fragmentations of Compounds **1**, **2**, and **3** in the Negative Ion Mode

compound	$[M - H]^-$	ESITQ-MS/MS m/z (% base peak)
1	565	CAD 565: 505(<5), 475(100), 403(45), 385(6), 355(7), 343(8), 337(5), 313(89), 295(<5), 271(<5) CAD 475:[a] 355(<5), 337(5), 313(100), 193(7)
2	563	CAD 563: 545(<5), 473(100), 417(7), 399(52), 381(5), 357(42), 339(10), 327(31), 309(11), 298(50), 285(8) CAD 473:[a] 327(56), 309(5), 298(100)
3	563	CAD 563: 545(<5), 473(100), 417(5), 399(25), 381(5), 357(21), 339(8), 327(13), 309(6), 298(23), 285(5) CAD 473:[a] 327(61), 309(<5), 298(100)

[a] In-source CAD.

The positive and negative ESI mass spectra showed ions at m/z 567, 589, and 565, corresponding to the $[M + H]^+$ and $[M + Na]^+$ adducts and to the $[M - H]^-$ ion, indicating a 566 Da molecular mass. In order to obtain structural information, collisionally activated dissociation (CAD) MS experiments were performed on deprotonated ions. Table 2 lists the CAD mass fragments observed for compound **1** and their relative abundancies. The $[M - H]^-$ CAD mass spectrum of compound **1** (m/z 565) showed fragments at m/z 475 and 505, deriving from the loss of 90 and 60 Da, which is typical of fragmentation of a pentose sugar bound to the aglycone through a C−C bond.[10,11] It can furthermore be hypothesized that the glycosylation position is at C-8, as no water loss, which is commonly observed for 6-*C*-glycosyl flavonoids, was observed.[10] This fragment with m/z 403 can be explained by the loss of 162 Da, which corresponds to the cleavage of a hexose glycosidic *O*-linkage with a concomitant H-rearrangement.[10] The fragment derived from a homolytic cleavage of the *O*-glycosidic bond, rendering a radical aglycone anion, was not detected in the spectrum. On the basis of previously published data,[10] this evidence is in agreement with a flavanone aglycone, as a 2,3-double bond adjacent to the 4-carbonyl in the C ring, required for the formation of stable radical product ions, is absent in a flavanone. Taking into account the molecular mass of compound **1** and the presence of both a pentose and a hexose moiety, the aglycone moiety has a molecular mass of 272 Da, which could reasonably correspond to naringenin. Confirming this hypothesis, ions at m/z 271 (deprotonated naringenin), 313, and 343 were detected. Considering the loss of a fraction of the *C*-glycosyl moiety, the ions at m/z 313 and 343 could correspond to $[\text{aglycone} + 41]^-$ or $[M - H - 162 - 90]^-$ and $[\text{aglycone} + 71]^-$ or $[M - H - 162 - 60]^-$ fragments, respectively.[11] The induced collisional dissociation of the ion at m/z 475, obtained by in-source CAD, gave a fragment at m/z 313 (−162 Da), which dominated the mass spectrum, as well as an ion with m/z 193 (−162 − 120 Da). The loss of 162 Da can be ascribed to the cleavage of an *O*-linked hexose, whereas the loss of 120 Da could be attributable to the cleavage of the C ring of the aglycone through an RDA mechanism, with the resulting loss of a neutral moiety containing the B ring.[10]

The HSQC/TOCSY experiment permitted assignment of the carbinol carbons of the pentose and hexose moieties. In fact, the anomeric proton at δ_H 4.35, bonded to the carbon at δ_C 74.0, also correlated with the methine carbons at δ_C 74.9, 69.0, and 68.0 and with the methylene carbon at δ_C 70.2, suggesting the presence of a pentose. As the anomeric carbon (δ_C 74.0) resonated in the δ_C 70−75 range, it could be deduced that the pentose is bound to the flavanone by a *C*-glycoside bond. The second anomeric proton at δ_H 4.68, bound to the carbon at δ_C 102.1 in the HSQC spectrum, correlated with the carbons at δ_C 77.6, 75.0, 73.3, 69.8, and 60.2, characteristic of a glucosyl unit.[12] The GC-MS analysis of the alditol acetate obtained after hydrolysis, reduction, and acetylation unequivocally identified the hexose as glucose, and the coupling constant ($J = 7.5$ Hz) of the glucosyl anomeric proton in **1** agreed with a β-configuration for an anomeric carbon *O*-linked to the aglycone. The pentose carbon data,[13] supported by correlations in



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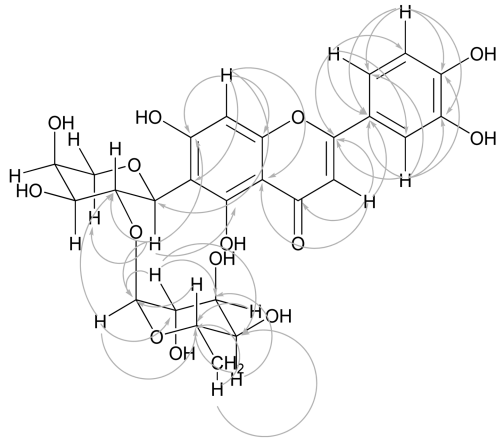

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Figure 2. Selected HMBC correlations of compounds **1** and **2**.

2D NMR experiments, were in good agreement with the presence of arabinose. The coupling constant of the arabinose anomeric proton ($J = 9.9$ Hz), as well as its chemical shift (δ 4.35), indicated an α-configuration for the anomeric carbon. The absolute D-configuration for glucose and L-configuration for arabinose were assumed from the natural occurrence of these sugar moieties in flavonoid glycosides.

The HMBC experiment (Figure 2) showed correlations between the anomeric proton at δ_H 4.35 and the carbons at δ_C 165.4, 107.7, and 160.2, attributable to the C-7, C-8, and C-9 flavanone carbons, respectively. The last carbon correlated with H-2 at δ_H 5.50, whereas H-2 showed cross-peaks with C-1′ and C-2′/6′. These data, together with the ^{13}C NMR shift of the arabinose anomeric carbon (δ_C 74.0), confirmed a *C*-glycoside linkage of the pentose to the C-8 carbon of naringenin. Finally, the glucose anomeric proton (δ_H 4.68) correlated to C-7 (δ_C 165.4), thus confirming the glucose moiety to be located at C-7 of the aglycone.

The UV spectrum showed two absorption regions: the first band was present at 332 nm; the second one, more intense, was at 286 nm. Likewise, in the CD spectrum two Cotton effects were observed: a weak positive Cotton effect at 332 nm ($\Delta\varepsilon$ +7.44), followed by a strong negative ($\Delta\varepsilon$ −45.54) Cotton effect at 286 nm. These data were in accordance with the *S*-configuration at C-2[14] of a flavanone.

The new compound **2** showed a molecular formula of $C_{26}H_{28}O_{14}$ according to the ESIMS and NMR data. In fact, the first-order ESI mass spectra showed ions at *m*/*z* 565, 587, and 563, corresponding to the $[M + H]^+$ and $[M + Na]^+$ adducts and to the $[M - H]^-$ ion, respectively. The molecular mass of compound **2** was thus concluded to be 564 Da. The ^{1}H NMR spectrum (Table 3) suggested the presence of a luteolin moiety as aglycone, as is evident from the presence of the H-2, H-5, and H-6 aromatic protons of the B ring at δ_H 7.37, 6.91, and 7.38, respectively, as well as singlets at δ_H 6.55 and 6.54. The ^{13}C NMR spectrum (Table 3) showed 26

Table 3. ^{1}H and ^{13}C NMR Data (300 MHz, methanol-d_4) of Compounds **2** and **3**

	2		**3**	
position	δ_C, mult.	δ_H (*J* in Hz)[a]	δ_C, mult.	δ_H (*J* in Hz)[a]
2	166.3, C		166.3, C	
3	103.8, CH	6.55, s	104.0, CH	6.56, s
4	184.0, C		184.1, C	
5	162.0, C		162.0, C	
6	109.6, C		109.5, C	
7	164.5, C		164.5, C	
8	96.0, CH	6.54, s	96.0, CH	6.51, s
9	158.8, C		158.8, C	
10	105.5, C		105.4, C	
1′	123.4, C		123.6, C	
2′	114.2, CH	7.37, overlap	114.1, CH	7.37, overlap
3′	151.0, C		151.0, C	
4′	147.0, C		147.1, C	
5′	116.9, CH	6.91, d (9.0)	116.8, CH	6.90, d (9.0)
6′	120.5, CH	7.38, overlap	120.3, CH	7.38, overlap
1-Pent	74.1, CH	4.80, obs	74.4, CH	4.79, obs
2-Pent	76.9, CH	3.75, overlap	72.4, CH	3.49, dd (8.7, 8.6)
3-Pent	71.2, CH	3.94, m	81.6, CH	3.96, m
4-Pent	75.9, CH	3.34, obs	73.6, CH	3.60, overlap
5-Pent	71.7, CH_2	4.02, m	71.5, CH_2	4.23, dd (8.1, 6.9)
		3.66, ov		3.36, obs
1-Rha	102.4, CH	5.15, d (1.2)	102.4, CH	5.21, brs
2-Rha	72.0, CH	3.89, m	71.1, CH	3.87, m
3-Rha	71.8, CH	3.44, dd (9.6, 2.7)	71.8, CH	3.43, dd (9.6, 2.7)
4-Rha	73.1, CH	3.13, dd (9.6, 9.3)	73.2, CH	3.12, dd (9.6, 9.3)
5-Rha	69.9, CH	2.52, dq (9.3, 6.3)	69.9, CH	2.55, m
6-Rha	16.8, CH_3	0.69, d (6.3)	16.8, CH_3	0.71, brs

[a] brs, broad singlet; d, doublet; dd, double doublets; dq, double quartets; m, multiplet; s, singlet; overlap, overlapped; obs, obscured.

signals identified as a methyl, a methylene, 14 methine, a carbonyl (δ_C 184.0), and nine quaternary carbons. The TOCSY and COSY experiments permitted identification of two different saccharidic proton spin systems. The anomeric proton at δ_H 5.15 correlated, in the DQCOSY spectrum, with a signal at δ_H 3.89, which in turn correlated with the doublet of doublets at δ_H 3.44. The latter showed cross-peaks with a doublet of doublets at δ_H 3.13, which in turn correlated with a doublet of quartets at δ_H 2.52, correlated to the methyl doublet at δ_H 0.69. These data and the corresponding carbon values obtained from an HSQC experiment indicated the presence of an *O*-linked L-rhamnose, later confirmed by GC-MS analysis of the alditol acetate from the hydrolysis, reduction, and acetylation of **2**. The second spin system consisted of an anomeric proton, obscured by the water signal at δ_H 4.80, bonded to the carbon at δ_C 74.1 (HSQC), and correlated with a signal at δ_H 3.75 (DQ-COSY), whereas the other protons overlapped in the saccharidic spectral region. The ^{13}C NMR spectrum of the *C*-glycoside obtained after the acidic hydrolysis of **2** suggested the presence of arabinose as the glycosyl moiety.[13]

The main CAD mass fragments observed for compound **2** and their relative abundances are listed in Table 2. The triple quadrupole CAD mass spectrum of compound **2**, $[M - H]^-$ ion at *m*/*z* 563, gave a fragment with *m*/*z* 473, corresponding to the loss of 90 Da. According to Cuyckens and Claeys,[10] this neutral loss is characteristic of a pentose or hexose residue linked to a flavonoid through a C−C bond. The absence of the fragment due to the loss of 120 Da and the high intensity of the ion at *m*/*z* 473 implied a pentose sugar bound to the C-6 position of the aglycone. The glycosylation position was confirmed by the number of water losses. The ion at *m*/*z* 399, formed after the loss of 164 Da (146 + 18 Da), could be indicative of a deoxyhexose residue bound to the pentose moiety at C-2″ through an *O*-glycosidic bond. The ions at *m*/*z* 357 ([aglycone + 71]$^-$), 339 ([aglycone + 71 − 18]$^-$), 327 ([aglycone + 41]$^-$), 309 ([aglycone + 41 − 18]$^-$), and 298, in the spectrum, led us to attribute a molecular mass of 286 Da to the aglycone, reasonably corresponding to luteolin. Confirming this hypothesis, the fragment at *m*/*z* 285 was detected in the spectrum. The ion at

m/z 473, obtained by in-source CAD, was allowed to undergo collision-activated dissociation, thus giving the fragment at *m/z* 327, probably due to the loss of the terminal deoxyhexose[10] (−146 Da) residue (Table 2).

The HMBC experiment (Figure 2) confirmed the hypothesized molecular structure. In fact, heterocorrelations between the anomeric proton at δ_H 4.80 and C-5, C-6, and C-7 aglycone carbons proved a C-6 glycosidic linkage. The anomeric proton also showed correlations with the C-2, C-3, and C-5 carbons of the arabinose. The C-2 carbon of arabinose (δ_C 76.9) correlated with the anomeric proton of rhamnose (δ_H 5.15), and, similarly, the C-1 carbon of rhamnose (δ_C 102.4) showed cross-peaks with the H-2 proton (δ_H 3.75) of arabinose, thus confirming the involvement of C-2 of arabinose and C-1 of rhamnose in an *O*-glycosidic linkage between the two sugar moieties. The H-1 proton of rhamnosyl coupling constant (J = 1.7 Hz) was in good accordance with that of the α-anomer,[12] while the J value of the pentose, obtained by a *J*-resolved experiment and compared to the literature data,[13] agreed with an α-anomer of L-arabinose.

Compound **3** was identified as an isomer of **2**. NMR data confirmed the presence of luteolin *C*-linked to the disaccharide moiety by the C-6 carbon with the NMR data being different from that of the pentose unit, in **2**. The pentose was identified as xylose by comparing the ^{1}H and ^{13}C NMR data registered in DMSO-d_6 and the literature.[13]

Given the positive and negative ESI mass spectra, which show ions at *m/z* 565, 587, and 563, corresponding to the $[M + H]^+$ and $[M + Na]^+$ adducts and to the $[M - H]^-$ ions, the molecular mass of compound **3** was concluded to be 564 Da. Table 2 lists the main CAD mass fragments observed for compound **3** and their relative abundancies. Only slight intensity differences were observed in the CAD mass spectra for compounds **2** and **3** (spectrum not shown), thus indicating that these luteolin derivatives could differ only with regard to the nature of sugar substituents, whereas their stereochemical characterization escapes the conventional MS techniques. HMBC correlations confirmed the hypothesized structure.

On the basis of NMR and MS results, compound **4** was characterized as isorientin, already reported as a constituent of *Sasa borealis*.[12,15]

Metabolite **5** was identified as 2″-*O*-rhamnosylisoorietin, and its structure confirmed by literature data.[16,17]

Compound **6**, also known as 2″-*O*-rhamnosylscoparin, was identified by comparing its NMR data with literature data.[18]

The NMR data of compound **7** suggested the presence of a 6-deoxyribohexos-3-ulose moiety linked by a *C*-glycosidic bond to C-6 of luteolin and were consistent with those of cassiaoccidentalin B, previously identified in *Cassia occidentalis*.[19]

The spectroscopic data of compound **8** agreed with those of an isomer of metabolite **7**. Differences regarded the 6-deoxyribohexosulose moiety, which showed oxidation at C-4 rather than at C-3. This compound had been identified as maysin, one of the principal components of *Zea mays*,[20] and the NMR data were consistent with those published.

Metabolites **1**−**6** from *P. velutina*, exhibiting the highest degree of pureness (99.9%), were tested for their potential antiproliferative capacity *in vitro* on human hepatocellular carcinoma HepG2 cells at different concentrations (50.0, 25.0, 12.5, and 6.25 μM). Cell proliferation was evaluated by determining the viable cells with the MTT assay. This method is based on the intracellular reduction of tetrazolium salts into a blue product called formazan by the mitochondrial enzyme succinate dehydrogenase. The activity was compared to that of catechin, tested at the same concentrations. The evaluation of mitochondrial redox activity showed that HepG2 cells exposed to the metabolites for 24 h are sensitive, even if slightly, to these molecules (Figure 3). A significative decrease of cell viability was observed for compound **4**, even at the lowest tested dose (6.25 μM), with an induced inhibitory effect of 19.6%.

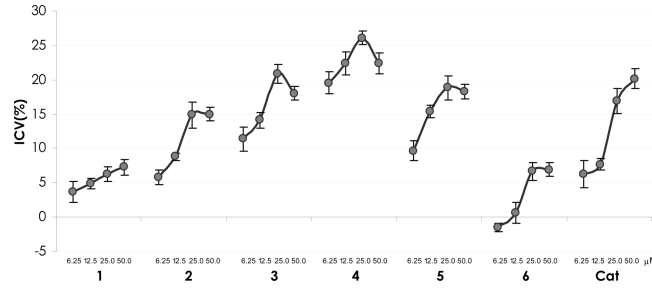

Figure 3. Cell viability inhibition (CVI, %) of metabolites **1**−**6** from *P. velutina* on HepG2 cells after 24 h treatment Values are reported as percentage ± SD vs an untreated control. Cat = catechin.

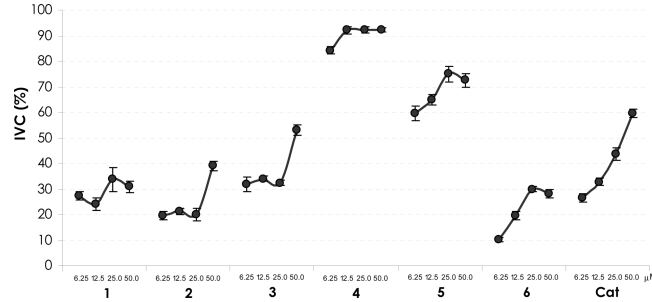

Figure 4. Cell viability inhibition (CVI, %) of metabolites **1**−**6** from *P. velutina* on HepG2 cells after 48 h treatment Values are reported as percentage ± SD vs an untreated control. Cat = catechin.

The inhibition increased almost linearly with the increase of the concentration until 25.0 μM, where the metabolite exerted an inhibiting capability of 26.1%. A weak antiproliferative effect was shown by compound **1**, a glycoside derivative of the flavanone naringenin, with a structure lacking the enone function of the C ring and cathechol moiety characterizing the B ring of luteolin, *C*-glycoside **4**, and metabolites **5**, **2**, and **3**. When the data of compounds **5**, **2**, and **3** are compared with those of isoorientin (**4**), the presence of a disaccharidic moiety seems to have a negative effect on the antiproliferative capability of the flavone. It is evident that compound **2** is less active than its isomer **3**, which suggests that the absolute configuration of a single carbon could modify the bioactivity of a substance. Data obtained suggested that loss in bioactivity is related to the number and type of saccharide moieties attached to the flavone.

Treatment of HepG2 cells for 48 h revealed a significant exposure time-dependent increase in the antiproliferative capacity of compound **4** (Figure 4). The molecule seemed to cause complete inhibition of the viability target cells, resulting in an antiproliferative efficacy of 84% at the lowest concentration tested. The activity increased to 92% when the metabolite concentration increased to 12.5 μM. Good reducing capacity for the tetrazolium salt was also observed for metabolite **5**, which showed an antiproliferative efficacy of 74.9% at a concentration of 25.0 μM. Compound **2** showed cytotoxic activity comparable to that shown by the catechin standard, which showed higher dose−response efficacy than the metabolites. The lower dose−response efficacy of the metabolites can probably be ascribed to the glycosidic nature of the isolated metabolites, since the presence of saccharide moieties might result in a decreased permeability of the complex cell system of the molecules.

The peculiar antiproliferative efficacy of some isolated metabolites is attributable to the molecular characteristics of the aglycone moiety. Literature data suggest that the *C*-glucoside isoorientin is a strong antiradical and antioxidant compound. Lim et al.[21] showed that HePG2 cells treated with isoorientin activates Nrf2 (NF-E2

related factor) and up-regulates antioxidant enzyme protein expression. The transcription factor Nrf2 is a central protein that interacts with the antioxidant response element (ARE) and regulates the transcription of genes encoding antioxidant proteins (Chan and Kan, 1999). Under physiological conditions, Nrf2 is sequestered in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (Keap1). Upon stimulation, Nrf2 dissociates from Keap1 and is translocated into the nucleus, where it binds to ARE, a control element present in the gene regions coding for antioxidant proteins, such as NAD(P)H quinone reductase (NQO1) and heme-oxigenase (HO-1). The researchers speculated that isoorietin could serve as a pro-oxidant since the translocation of Nrf2 to the nucleus is sensitive to cellular oxidative stress. In fact, recent studies have shown that certain polyphenols can act as antioxidants or pro-oxidants depending on their concentration, the source of free radicals, and specific intracellular conditions but mainly on the presence of *o*-dihydroxylation of the flavone aromatic B ring. Flavones with this substitution can easily autoxidize to quinones, compounds able to induce a strong expression of antioxidant enzymes via ARE. *In vitro* and *in vivo* studies have demonstrated the ability of luteolin and other flavonoids to inhibit carcinogenesis by acting on the processes of cancer initiation and progression. It has shown that some flavonoids modulate the metabolism and disposition of carcinogens and can contribute to cancer prevention. One important mechanism by which flavonoids may exert their effects is through their interaction with phase I metabolizing enzymes (e.g., cytochrome P450), which metabolically activate a large number of procarcinogens to reactive intermediates able to interact with cellular nucleophiles and ultimately trigger carcinogenesis. Flavonoids have been shown to inhibit the activities of certain P450 isozymes such as CYP1A1 and CYP1A2. Thus, they are likely to have a protective role against the induction of cellular damage by the activation of carcinogens.[22] Another mechanism of action is the induction of phase II metabolizing enzymes such as glutathione-S-transferase, quinone reductase, and UDP-glucuronyl transferase, whereby carcinogens are detoxified and thus more readily eliminated from the body. This would also help explain the chemopreventive effects of flavonoids against carcinogenesis.[23]

The efficacy of flavonoids as anticancer agents appears to be largely linked to their ability to induce apoptosis in cancer cells through multiple molecular mechanisms including the inhibition of the enzyme DNA topoisomerase I and II, the decrease of the reactive oxygen species (ROS) concentration, the activation of the heat shock protein expression, and the modulation of different intracellular signaling cascades.

The current research interest in relation to flavonoids is motivated by the strong versatility of the ubiquitous flavonoid skeleton and by an increased awareness of the benefits they have or could have on the human body. The phytochemical analysis of the methanolic extract of the leaves of *P. velutina* led to the isolation of eight *C*-glycoside flavonoids, three of them isolated for the first time and identified by NMR and ESIMS experiments. The evaluation of the antiproliferative capacity of isolated compounds has highlighted the peculiar efficacy of metabolites whose molecular skeleton is characterized by the presence of the luteolin aglycone. Isoorietin **4** significantly reduces the cell proliferation of HepG2 cells, as evidenced by the complete conversion of the tetrazolium probe into formazan after 48 h of exposure. The data obtained also emphasize the importance of some structural variants in flavonoid skeleton on their bioactivity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 in MeOH solution. UV spectra were performed on a UV-1700 Shimadzu spectrophotometer in MeOH solution. CD spectra were recorded on a Jasco J-815 CD spectrometer in MeOH solution. IR spectra were determined in the KBr pellet using a FT-IR Perkin-Elmer Spectrum GX spectrometer. NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian 300 Fourier transform instrument in methanol-d_4 or DMSO-d_6 (plus 2 drops of D_2O) at 25 °C. TOCSY experiments were performed in the phase-sensitive mode with a mixing time of 90 ms. NOESY experiments were performed in the phase-sensitive mode. The mixing time was 500 ms, and the spectral width was 3000 Hz. Proton-detected heteronuclear correlations were measured using a gradient HSQC, optimized for $^1J_{HC}$ = 140 Hz, and a gradient HMBC, optimized for $^nJ_{HC}$ = 8 Hz. The HSQC experiment was performed in the phase-sensitive mode with field gradient. 2D *J*-resolved spectra were acquired using 160 scans per 256 increments for F1 and 2K for F2 using spectral widths of 2420 Hz in F2 (chemical shift axis) and 50 Hz in F2 (spin spin coupling constants axis). Electrospray-ionization mass spectra (ESIMS) [Quattro Micro (Micromass)] were recorded on a triple quadrupole instrument equipped with an ESI source operating in negative or positive ion mode; N_2 was used as nebulization and desolvation gas at flow rates of 70 and 700 L/h, respectively. Source and desolvation temperatures were set at 120 and 350 °C, respectively. Applied potential on the ES capillary and on the cone ranged from 2.8 to 3.2 kV and from 15 to 55 V, respectively, and were optimized for each molecule. CAD/MS experiments were performed with Ar as collision gas at a pressure of (3.0−5.0) × 10^{-3} mbar with collision energy varying from 20 to 40 eV. All mass spectra were performed by direct infusion of purified compounds (10^{-4} M) in MeOH/H_2O (1:1, v/v), with a 10 μL/min flow rate. Full-scan mass spectra were acquired from *m*/*z* 70 to 1000 in MS and from 20 to the *m*/*z* ratio of the precursor ion in MS/MS experiments, with a scan time of 0.2 s.

The preparative HPLC apparatus consisted of a Knauer Smartline 31/40 module equipped with a Knauer Smartline 1000 pump, a UV Knauer Smartline 2500 detector, an RI Knauer Smartline 2300 detector, and PC CromGate software. Preparative HPLC were performed using Luna RP-8 (10 μm, 250 × 10.0 mm i.d., Phenomenex) and Luna RP-18 (10 μm, 250 × 10.0 mm i.d., Phenomenex) columns. Analytical TLC were performed on Merck Kieselgel 60 F_{254} or RP-8 F_{254} plates with a 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4/AcOH/H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with a 0.5 or 1.0 mm film thickness. Column chromatography (CC) was performed on Merck Kieselgel 60 (70−240 mesh), Merck Kieselgel 60 (40−63 μm), RP silica gel 100 C8 (40−63 μm; Fluka), Amberlite XAD-4 (20−50 mesh; Fluka), Amberlite XAD-7 (20−50 mesh; Fluka), and Sephadex LH-20 (Pharmacia).

Human hepatoblastoma cells (HepG2) were purchased from ICLC (Interlab Cell Line Collection) at Istituto Nazionale per la Ricerca sul Cancro, Genoa (Italy). HepG2 were grown in RPMI containing 10% fetal bovine serum, 50 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2.

GC-MS qualitative analyses were carried out using a HP 6890 GC instrument equipped with a 5975B VL MSD detector. The GC was equipped with a fused silica capillary column (Zebron ZB5MS, 30 m × 0.25 mm i.d., film thickness 0.25 μm), with He as carrier gas (flow 0.6 mL/min). The column head pressure was set at 7.41 psi. Temperature conditions were as follows: injector port at 250 °C; initial oven temperature 160 °C for 50 s, then increased linearly to 200 at 10 °C/min, again increased linearly at 2.5 °C/min to 300 °C, and held for 40 min. Sample solutions were injected using the split mode. Full-scan mass spectra were collected between 0 and 600 amu at 2 scan/s. The MS was operated in the electron impact (EI) ionization mode with an electron energy of 70 eV. The ion source and quadrupole temperatures were maintained at 230 and 150 °C, respectively.

Plant Material. Plants of *Petrorhagia velutina* (Guss.) Ball. et Heyw. (Caryophyllaceae) were collected in June 2007, in the vegetative state, at the Natural Reserve of Castel Volturno (Caserta, Italy) and identified by Dr. Assunta Esposito of the Second University of Naples. A voucher specimen (CE 0115) has been deposited in the Herbarium of the Dipartimento di Scienze della Vita of Second University of Naples.

Extraction and Isolation. Dried plants of *P. velutina* (670.7 g) were extracted consecutively in the dark at 4 °C with petroleum ether (PE), EtOAc, and finally MeOH for three days each. After the removal of the solvent by Rotavapor, PE (3.0 g), EtOAc (13.0 g), and MeOH (31.4 g) crude extracts were obtained.

The methanolic extract, dissolved in H_2O, was separated by liquid−liquid extraction using EtOAc as solvent. The aqueous fraction was chromatographed on Amberlite XAD-4 and Amberlite XAD-7, successively. The MeOH eluate obtained from Amberlite XAD-7 (3.49

g) was chromatographed on Sephadex LH-20 using H_2O/MeOH with decreasing polarity. Four fractions (A−D) were obtained. Fraction A (120 mg), chromatographed by RP-8 HPLC using H_2O (TFA, 0.1%)/MeOH/MeCN (6:3:1), furnished pure compounds **5** (71.8 mg), **2** (37.2 mg), **3** (2.8 mg), **7** (3.3 mg), and **8** (2.2 mg). Fraction B (30.0 mg) was analogously chromatographed giving pure metabolite **4** (6.1 mg). Fraction C (140.0 mg) was chromatographed by RP-8 CC eluting with H_2O/MeOH solutions. One of the obtained fractions (51.8 mg) gave pure compound **4** (17.6 mg) after TLC (1.0 mm) with the organic lower phase of a $CHCl_3$/MeOH/H_2O (13:9:3) biphasic solution as eluent,. Fraction D (467.0 mg) was chromatographed by RP-8 CC eluting with H_2O/MeOH solutions. One of the obtained fractions (81.1 mg), chromatographed by RP-18 HPLC with a H_2O (TFA, 0.1%)/MeOH/MeCN (13:5:2) solution as eluent, gave pure compound **1** (4.1 mg).

8-*C*-α-L-Arabinopyranosyl-7-*O*-β-D-glucopyranosylnaringenin (1): light yellow oil, $[\alpha]^{20}_D$ −36.7 (*c* 0.84, MeOH); UV (MeOH) λ_{max} (log ε) 332 (3.29), 284 (3.92) nm; CD (MeOH) $\Delta\varepsilon$ (nm) +7.44 (332), −45.54 (286), +4.68 (250), +38.08 (216); IR ν_{max} (KBr) cm^{-1} 3881, 3046, 1635, 1594, 1478; 1D and 2D NMR data (DMSO-d_6) see Table 1; ESIMS *m*/*z* 589 $[M + Na]^+$; 567 $[M + H]^+$; 565 $[M - H]^-$ see Table 2; *anal*. calcd for $C_{26}H_{30}O_{14}$, C, 53.61; H, 5.19%, found C, 53.63; H, 5.16%.

6-*C*-[2′-*O*-α-L-Rhamnopyranosyl-(1″→2′)]-α-L-arabinopyranosylluteolin (2): $[\alpha]^{20}_D$ +4.0 (*c* 0.69, MeOH); UV (MeOH) λ_{max} (log ε) 350 (3.84), 270 (3.78) nm; IR ν_{max} (KBr) cm^{-1} 3880, 3048, 1655; ^1H and ^{13}C NMR data (methanol-d_4) see Table 3; ESIMS *m*/*z* 587 $[M + Na]^+$; 565 $[M + H]^+$; 563 $[M - H]^-$ see Table 2; *anal*. calcd for $C_{26}H_{28}O_{14}$, C, 55.32; H, 5.00%, found C, 55.34; H, 4.97%.

6-*C*-[2-*O*-α-L-Rhamnopyranosyl-(1″→2′)]-β-D-xylopyranosylluteolin (3): $[\alpha]^{20}_D$ −12.8 (*c* 0.45, MeOH); UV (MeOH) λ_{max} (log ε) 314 (3.48), 260 (3.55) nm; IR ν_{max} (KBr) cm^{-1} 3879, 3050, 1652; ^1H and ^{13}C NMR data (methanol-d_4) see Table 3; ESIMS: *m*/*z* 587 $[M + Na]^+$; 565 $[M + H]^+$; 563 $[M - H]^-$ see Table 2; *anal*. calcd for $C_{26}H_{28}O_{14}$, C, 55.32; H, 5.00; found C, 55.35; H, 5.02.

GC-MS Analysis of the Sugar Moieties. Saccharidic moieties were detected by comparing the retention times and the fragmentation patterns from the EIMS spectra of acetylated alditol derivatives of isolated metabolites with those of eight monose standards subjected to similar prior derivatization and GC-MS analysis.

Each metabolite (0.5 mg) was subjected to an acid hydrolysis with 2 N TFA (150 μL). The hydrolysis reaction was conducted at 120 °C for 1 h. The reaction mixture was then suitably dried under N_2 flow. The reduction of the sugar moiety was achieved by dissolving the sample in MeOH (150 μL) and adding $NaBH_4$ (1.0 mg). The solution was incubated at room temperature for 1 h and then dried under N_2 flow after treatment with glacial AcOH and MeOH. Finally, the obtained alditol was acetylated in anhydrous pyridine (200 μL) and Ac_2O (200 μL). After 20 min at 120 °C 500 μL of H_2O was added, and the product was extracted with CH_2Cl_2 (500 μL) in a centrifuge (3500 rpm for 5 min). The organic phase was dried under nitrogen flow, dissolved in CH_2Cl_2 (500 μL), and analyzed by GC-MS.

Acid Hydrolysis of Compound 2. Compound **2** (20.0 mg) was dissolved in 500 μL of 2 N TFA. The hydrolysis reaction was conducted at 120 °C for 1 h. The reaction mixture was then suitably dried under N_2 flow. The hydrolysis product was purified by preparative TLC using the organic lower phase of a $CHCl_3$/MeOH/H_2O (13:7:2) biphasic solution as eluent. ^1H NMR (methanol-d_4, 300 MHz) δ_H 7.34 (2H, ov, H-2′, H-6′), 6.92 (1H, d, J = 8.9 Hz, H-5′), 6.50 (1H, s, H-3), 6.46, (1H, s, H-8), 4.82 (1H, d, J = 8.8 Hz, H-1Ara), 4.25 (1H, dd, J = 8.8, 8.5 Hz, H-2Ara), 4.2−3.7 (4H, ov, saccharidic protons) 3.63 (1H, dd, J = 9.6, 3.0 Hz, H-5Ara); ^{13}C NMR (75 MHz) δ_C 184.1 (C-4), 166.4 (C-2), 164.8 (C-7), 161.5 (C-5), 158.8 (C-9), 151.1 (C-3′), 147.1 (C-4′), 123.6 (C-1′), 120.5 (C-6′), 116.9 (C-5′), 114.3 (C-2′), 109.4 (C-6), 105.3 (C-10), 104.0 (C-3), 95.2 (C-8), 74.4 (C-3Ara), 73.7 (C-1Ara), 70.8 (C-5Ara), 69.4 (C-4Ara), 68.0 (C-2Ara).

Antiproliferative Activity. The MTT test allowed cell viability to be assessed by determining the levels of activity of mitochondrial dehydrogenases toward 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT).[24] The assay is based on the mitochondrial redox capacity to convert MTT into an insoluble blue product called formazan. HepG2 cells were seeded in 96-multiwell plates at a density of 4×10^5 cells/well. After 24 h of incubation, cells were treated with test compounds at four concentrations (50.0, 25.0, 12.5, and 6.25 μM). At 24 and 48 h of incubation, cells were treated with 200 μL of 5.0 mg/mL MTT, dissolved in the culture medium, for 1 h at 37 °C in a 5% CO_2 humidified atmosphere. The MTT solution was then removed, and 100 μL of DMSO was added to solubilize the formazan formed. Finally, the absorbance at 570 nm of each well was determined using a BioRad 680 microplate reader. Cell viability was then expressed as percentage of the mitochondrial redox activity of the cells treated with the metabolites compared to the untreated control. Inhibition of cell viability (ICV, %) was calculated using the following formula: (absorbance of untreated cells) − (absorbance of treated cells)/(absorbance of untreated cells) × 100.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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