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NEW ISORHAMNETIN GLYCOSIDES AND OTHER PHENOLIC COMPOUNDS FROM *Calendula officinalis*

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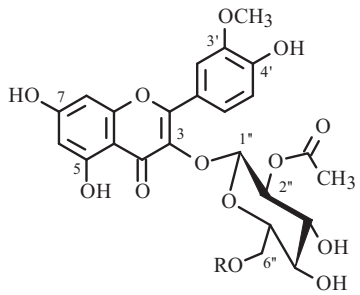
*A total of 39 known compounds and two new flavonoid glycosides that were identified as isorhamnetin-3-*O*-(2″-acetyl)-*β*-D-glucopyranoside and isorhamnetin-3-*O*-(2″,6″-diacetyl)-* β*-D-glucopyranoside were isolated from florets of* Calendula officinalis *(Asteraceae). The distribution of phenolic compounds in morphological groups of* C. officinalis *was studied. It was found that peripheral florets had the highest flavonoid content (36.66 mg/g); tubular florets (9.95 mg/g) and leaves (9.65 mg/g), phenylpropanoids. Anthocyanins, among which cyanidin derivatives dominated, were identified for the first time in* C. officinalis *florets.*

Keywords: *Calendula officinalis*, Asteraceae, isorhamnetin-3-*O*-(2″-acetyl)-*β*-D-glucopyranoside, isorhamnetin-3-*O*-(2″,6″-diacetyl)-*β*-D-glucopyranoside, flavonoids, anthocyanins, HPLC.

Calendula officinalis L. is an annual herbaceous plant of the family Asteraceae that is widely used in medical practice. Chemical studies found in *C. officinalis* various classes of compounds including terpenes, phenols, lipids, carbohydrates, etc. [1]. *C. officinalis* is a cultivated species that is remarkable for its adaptability that enables it to be grown even in inhospitable agricultural zones, in particular, in the Transbaikal territory. Several double-flowered varieties (*Big Orange*, *Egypt Sun*, *Flame Dancer*, *Geisha Girl*, *Indian Prince*, *Radio*, *Red Black Centered*) were recommended for cultivation of *C. officinalis* in the Republic of Buryatiya. These are characterized by high productivity and a prolonged vegetative period. Information on the chemical compositions of these varieties has not been published. Therefore, the goal of the present work was to study phenolic compounds from seven *C. officinalis* varieties introduced into the Republic of Buryatiya.

Preliminary studies of florets of the studied *C. officinalis* varieties for the presence of the principal compound groups showed that the contents of essential oil, carotenoids, and water-soluble polysaccharides in them were 1.4–2.9, 5.14–7.59, and 14.51–28.81 mg/g, respectively (Table 1). The total flavonoid concentration varied from 10.52 (*Flame Dancer*) to 26.79 mg/g (*Big Orange*), which was greater than that for raw material grown in the central part of Russia (2.6–9.1 mg/g) [2], Estonia (2.1–6.8 mg/g) [3], Italy (2.8–7.5 mg/g) [4], and Brazil (14.1–14.4 mg/g) [5]. Then, we studied the phenolic components from florets of *Big Orange* variety, from which fractionation and chromatographic separation of the extracted compounds identified 41 compounds including 39 known compounds and two new flavonoids **1** and **2**.



1, 2

1: R = H; **2:** R = Ac

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TABLE 1. Chemical Composition of Florets of *C. officinalis* Varieties, mg/g*

Compound group	*Big Orange*	*Egypt Sun*	*Flame Dancer*	*Geisha Girl*	*Indian Prince*	*Radio*	*Red Black Centered*
Flavonoids	26.79	19.24	10.52	20.11	17.25	18.42	16.34
Essential oil	2.6	1.8	2.9	2.5	1.4	1.8	2.1
Carotenoids	6.56	5.47	7.59	6.97	5.14	5.98	6.77
Polysaccharides	14.63	17.45	24.48	10.28	28.81	15.51	14.51

*Of air-dried raw material mass.

TABLE 2. PMR (500 MHz) and ^{13}C NMR (125 MHz) Spectra of **1** and **2** (DMSO-d_6, δ, ppm, J/Hz)

C atom	DEPT	**1**		**2**	
		δ_H	δ_C	δ_H	δ_C
			Isorhamnetin		
2	C		156.4		156.4
3	C		133.9		134.0
4	C		178.3		178.2
5	C		161.0		161.2
6	CH	6.15 (1H, d, J = 2.2)	99.9	6.14 (1H, d, J = 2.2)	99.8
7	C		164.2		164.3
8	CH	6.33 (1H, d, J = 2.2)	93.8	6.33 (1H, d, J = 2.2)	93.4
9	C		156.1		156.1
10	C		104.1		104.2
1′	C		121.7		121.9
2′	CH	7.86 (1H, d, J = 2.0)	112.9	7.85 (1H, d, J = 2.0)	112.9
3′	C		149.2		149.0
4′	C		146.4		146.3
5′	CH	6.89 (1H, d, J = 9.0)	115.7	6.87 (1H, d, J = 9.0)	115.9
6′	CH	7.45 (1H, d, J = 9.0, 2.0)	122.5	7.44 (1H, dd, J = 9.0, 2.0)	122.4
3′-OCH_3	CH_3	3.81 (3H, s)	55.2	3.82 (3H, s)	55.3
			β-D-Glucopyranose		
1″	CH	5.81 (1H, d, J = 7.7)	101.7	5.83 (1H, d, J = 7.7)	101.8
2″	CH	4.58 (1H, dd, J = 8.1, 8.6)	75.9	4.60 (1H, dd, J = 8.1, 8.6)	76.0
3″	CH		77.9		78.0
4″	CH	3.11–3.35 (3H, m)	70.2	3.15–3.36 (3H, m)	70.2
5″	CH		76.3		76.7
6″	CH_2	3.75 (1H, m, H_A), 3.51 (1H, m, H_B)	61.1	4.25 (1H, dd, J = 2.0, 12.0, H_A), 4.09 (1H, m, H_B)	63.9
2″- $CO\underline{CH_3}$	CH_3	1.59 (3H, s)	19.6	1.57 (3H, s)	19.7
2″- $\underline{C}OCH_3$	C		170.2		170.2
6″- $CO\underline{CH_3}$	CH_3			1.63 (3H, s)	20.3
6″- $\underline{C}OCH_3$	C				171.0

Compound **1** was a yellow amorphous powder of formula $C_{24}H_{24}O_{13}$ according to HR-ESI-MS data {*m/z* 543.421 ($[M + Na]^+$; calcd 543.441)}. The IR spectrum contained bands for ester carbonyl (1725 cm^{-1}), which indicated that **1** was acylated. Isorhamnetin and glucose were identified in the acid hydrolysis products. Alkaline hydrolysis produced isorhamnetin-3-*O*-β-D-glucopyranoside and acetate, which was identified by HPLC. Isorhamnetin was detected after enzymatic hydrolysis of **1** by β-glucosidase. This was consistent with the presence of an acetyl in the carbohydrate part of the glycoside. PMR and ^{13}C NMR spectroscopic data confirmed the β-configuration of the anomeric center of the glucopyranose. The PMR spectrum contained a resonance for an anomeric proton of glucopyranose as a doublet at 5.81 ppm (1H, J = 7.7 Hz); the ^{13}C NMR spectrum, a resonance at 101.7 ppm (Table 2). Also, the PMR spectrum exhibited a singlet for acetyl at 1.59 ppm. The presence of a 1H doublet of doublets at 4.58 ppm indicated that a glucopyranose secondary hydroxyl was acetylated (C-2″, C-3″, C-4″) because the resonance would have appeared at stronger field if the C-6″ primary hydroxyl was acetylated, like for isorhamnetin-3-*O*-(6″-acetyl-)-β-D-glucopyranoside (**30**) [6]. The C-2″ resonance of the carbohydrate of **1** underwent a weak-field shift (+2.8 ppm) according to ^{13}C NMR spectroscopy. This was due to acylation at this position. The resonance of the acetyl carbonyl (170.2 ppm) in the HMBC spectrum correlated with proton H-2″ of the glucopyranose (4.58 ppm), confirming that C-2″ was acetylated. The studies established the structure of **1** as isorhamnetin-3-*O*-(2″-acetyl-)-β-D-glucopyranoside.

TABLE 3. Antioxidant Activity of Quercetin and Isorhamnetin Glycosides, IC_{50}, μg/mL[a]

Glycoside part	DPPH[b]		CBA[c]	
	Quercetin	Isorhamnetin	Quercetin	Isorhamnetin
–	9.5	14.31	6.02	9.95
3-*O*-Glc	21.29	62.14	17.24	37.14
3-*O*-(2″-Acetyl-)-Glc	63.12	> 100	35.28	85.84
3-*O*-(6″-Acetyl-)-Glc	57.82	> 100	36.02	80.23
3-*O*-(2″,6″-Diacetyl-)-Glc	71.39	> 100	42.11	> 100

[a]n = 5; [b]antiradical activity relative to DPPH•; [c]peroxide degradation of *β*-carotene.

TABLE 4. Contents of Phenolic Compounds in Morphological Groups of *C. officinalis*, mg/g[a,b]

Compound, compound group	Peripheral florets	Tubular florets	Spathes	Seeds	Leaves	Stems	Roots
Caffeic acid (**12**)	0.09	0.49	0.09	Tr.	0.42	0.05	0.31
3-*O*-Caffeylquinic acid (**13**)	1.34	6.91	0.89	2.11	8.46	0.23	0.28
4-*O*-Caffeylquinic acid (**31**)	–	0.31	0.13	–	0.77	0.03	0.83
5-*O*-Caffeylquinic acid (**14**)	Tr.	Tr.	0.10	Tr.	Tr.	0.05	1.36
1,3-di-*O*-Caffeylquinic acid (**15**)	Tr.	Tr.	–	0.16	–	–	0.12
3,5-di-*O*-Caffeylquinic acid (**17**)	0.54	1.78	0.45	0.82	Tr.	0.01	0.22
4,5-di-*O*-Caffeylquinic acid (**18**)	0.07	0.46	0.11	0.21	–	0.02	3.34
Manghaslin (**37**)	1.22	0.29	–	–	–	0.11	–
Calendoflavobioside (**36**)	0.90	0.35	–	–	1.42	0.65	–
Rutin (**33**)	2.28	0.34	–	–	–	0.10	–
Quercetin-3-*O*-(2″-Rha-)-Rha (**32**)	Tr.	0.46	–	–	Tr.	–	–
Isoquercitrin (**23**)	Tr.	0.24	–	–	3.28	0.45	–
Quercetin-3-*O*-(6″-acetyl)-Glc (**24**)	0.63	1.04	–	–	3.04	0.11	–
Typhaneoside (**38**)	8.95	7.85	0.06	Tr.	–	0.13	–
Calendoflavoside (**35**)	1.29	1.63	–	–	–	0.19	–
Narcissin (**34**)	14.62	4.53	–	–	–	0.05	–
Calendoflaside (**29**)	0.43	0.61	–	–	–	–	–
Isorhamnetin-3-*O*-Glc (**27**)	1.71	2.14	–	–	0.38	0.16	–
Isorhamnetin-3-*O*-(6″-acetyl)-Glc (**30**)	4.63	4.09	–	–	1.04	0.04	–
Identified, including:	38.70	33.52	1.83	3.30	18.81	2.38	6.46
phenylpropanoids	2.04	9.95	1.77	3.30	9.65	0.39	6.46
flavonoids, including:	36.66	23.57	0.06	Tr.	9.16	1.99	–
quercetin derivatives	5.03	2.72	–	–	7.74	1.42	–
isorhamnetin derivatives	31.63	20.85	0.06	Tr.	1.42	0.57	–

[a]Of air-dried raw material mass; [b]variety *Big Orange*. Tr.: traces.

Compound **2** was a yellow amorphous powder of molecular formula $C_{26}H_{26}O_{14}$ {HR-ESI-MS *m*/*z* 585.263 ([M + Na]$^+$; calcd 585.479)}. Chemical transformations and UV, IR and NMR spectroscopy were similar to those of **1** and isorhamnetin-3-*O*-(6″-acetyl-)-*β*-D-glucopyranoside (**30**) with the exceptions that the PMR spectrum contained two acetyl resonances at 1.57 and 1.63 ppm and the ^{13}C NMR spectrum exhibited chemical shifts for carbonyl at 170.2 and 171.0 ppm and methyl at 19.7 and 20.3 ppm (Table 2). These facts indicated that two acetyls were present in the structure of **2**. Correlations between the acetyl carbonyls at 170.2 and 171.0 ppm and resonances of protons H-2″ (4.60 ppm) and H-6″ (4.09 and 4.25 ppm) were found. This established that the acetyls were located on C-2″ and C-6″ of *β*-glucopyranose. Thus, the structure of **2** was determined as isorhamnetin-3-*O*-(2″,6″-diacetyl-)-*β*-D-glucopyranoside.

Two acetylated derivatives of isorhamnetin-3-*O*-*β*-D-glucoside, i.e., isorhamnetin-3-*O*-(6″-acetyl-)-*β*-D-glucopyranoside, which was isolated from *Pinus silvestris* L. (Pinaceae) [6], and isorhamnetin-3-*O*-(2″,3″,4″-triacetyl-)-*β*-D-glucopyranoside from leaves of *Warburgia stuhlmanii* Engl. (Canellaceae) [7], are known. Compounds **1** and **2** were new natural compounds.

A comparison of the antioxidant activity of 3-*O*-glucosides of quercetin and isorhamnetin showed that introducing an acetyl into the glycoside part of the flavonoid reduced the activity (Table 3). The activities of the isorhamnetin derivatives were significantly less than those of the quercetin derivatives.

TABLE 5. Contents of Phenolic Compounds in Florets of *C. officinalis* Varieties, mg/g[a]

Compound, compound group	*Big Orange*	*Egypt Sun*	*Flame Dancer*	*Geisha Girl*	*Indian Prince*	*Radio*	*Red Black Centered*
12	0.17	0.06	0.20	0.14	0.06	0.11	0.08
13	2.09	0.63	5.77	3.00	1.81	1.66	2.49
37	1.80	1.14	1.12	0.96	0.66	1.29	0.83
36	1.96	1.10	1.26	1.32	0.98	1.61	1.12
33	3.72	1.23	0.66	1.49	0.95	1.52	1.15
32	0.29	0.28	0.06	0.32	0.23	0.24	0.21
23	0.30	0.06	0.11	0.20	0.15	0.11	0.22
24	0.61	0.33	Tr.	0.62	0.23	0.18	0.46
38	5.01	4.84	2.22	4.74	3.76	4.06	2.93
35	1.27	1.10	0.46	1.05	0.82	0.94	0.67
34	8.52	4.60	2.10	4.57	3.53	5.04	4.13
29	0.15	0.17	0.12	0.25	0.23	0.18	0.23
27	0.98	0.59	0.42	0.85	0.79	0.62	0.81
30	0.69	1.80	1.22	1.64	3.27	1.62	1.59
Identified, including:	27.56	17.93	15.72	21.24	17.47	19.18	16.92
phenylpropanoids	2.26	0.69	5.97	3.14	1.87	1.77	2.57
flavonoids, including:	25.30	17.24	9.75	18.10	15.60	17.41	14.35
quercetin derivatives	8.68	4.14	3.21	4.91	3.20	4.95	3.99
isorhamnetin derivatives	16.62	13.10	6.54	13.10	12.40	12.46	10.36
Total content[b]:							
flavonoids	26.79	19.24	10.52	20.11	17.25	18.42	16.34
anthocyanins	–	2.29	3.12	–	0.79	–	2.48

[a]Of air-dried raw material mass; [b]spectrophotometric method. Tr.: traces.

The known compounds that were isolated from florets of *C. officinalis* also included phenolic acids such as vanillic (**3**) and syringic (**4**); phenylpropanoids such as cinnamic (**5**), *p*- (**6**) and *o*-coumaric (**7**), ferulic (**8**), isoferulic (**9**), caffeic acids (**12**), 1-*O*- (**40**), 3-*O*- (**13**), 4-*O*- (**31**), 5-*O*- (**14**), 1,3-di-*O*- (**15**), 3,4-di-*O*- (**16**), 3,5-di-*O*- (**17**), 4,5-di-*O*- (**18**), 1,3,5-tri-*O*- (**19**), and 3,4,5-tri-*O*-caffeylquinic acids (**20**); 3-*O*-*p*-coumarylquinic acid (**41**), 5-*O*-ferulylquinic acid (**21**), 1-*O*-caffeylglucose (**39**); flavonoids such as quercetin (**10**), isorhamnetin (**11**), quercitrin (**22**), isoquercitrin (**23**), quercetin-3-*O*-(2″-acetyl-)-*β*-D-glucopyranoside (**24**), quercetin-3-*O*-(6″-acetyl-)-*β*-D-glucopyranoside (**25**), quercetin-3-*O*-(2″,6″-diacetyl-)-*β*-D-glucopyranoside (**26**), isorhamnetin-3-*O*-*β*-D-glucopyranoside (**27**), isorhamnetin-3-*O*-*α*-L-rhamnopyranoside (**28**), calendoflaside (**29**), isorhamnetin-3-*O*-(6″-acetyl-)-*β*-D-glucopyranoside (**30**), quercetin-3-*O*-(2″-*α*-L-rhamnopyranosyl)-*α*-L-rhamnopyranoside (**32**), rutin (**33**), narcissin (**34**), calendoflavoside (**35**), calendoflavobioside (**36**), manghaslin (**37**), and typhaneoside (**38**). Compounds **3**, **4**, **6**, **8**, **10**–**13**, **22**, **27**, **29**, and **33**–**38** were identified earlier in *C. officinalis* [8–11]. Compounds **5**, **7**, **9**, **14**–**21**, **23**–**26**, **28**, **30**–**32**, and **39**–**41** were detected in it for the first time.

According to HPLC data, peripheral (36.66 mg/g) and tubular florets (23.57 mg/g) of *C. officinalis* typically had the greatest contents of flavonoids. These morphological groups were dominated by isorhamnetin derivatives narcissin (**34**), typhaneoside (**38**), and isorhamnetin-3-*O*-(6″-acetyl-)-*β*-D-glucopyranoside (**30**). The contents of quercetin glycosides were less than 15% of the total flavonoids (Table 4). Trace concentrations of flavonoids were found in spathes and seeds. Quercetin derivatives isoquercitrin (**23**), quercetin-3-*O*-(2″-acetyl-)-*β*-D-glucopyranoside (**24**), and calendoflavobioside (**36**) predominated in flavonoids from leaves and stems. This group of compounds was not observed in roots.

Phenylpropanoids were represented by free caffeic acid (**12**) in addition to mono- and dicaffeylquinic acids, which were detected in all morphological groups. A feature of the aerial organs was the dominance of monocaffeylquinic acids (1.34–9.23 mg/g) and 3-*O*-caffeylquinic acid (**13**) as the principal compound (0.23–8.46 mg/g). A high concentration of dicaffeylquinic acids (3.68 mg/g) was detected in roots. The dominant component was 4,5-di-*O*-caffeylquinic acid (**18**, 3.34 mg/g).

A study of the accumulation of pure phenolic compounds of *C. officinalis* in different varieties showed that marker components (**12**, **13**, **23**, **24**, **27**, **29**, **30**, **32**–**38**) were typically present for all studied varieties (Table 5). The ratios of the contents of individual compounds for different varieties were similar. However, it should be noted that the principal flavonoid of varieties *Big Orange*, *Radio*, and *Red Black Centered* was narcissin (**34**, 4.13–8.52 mg/g); for *Egypt Sun*, *Flame Dancer*, *Geisha Girl*, and *Indian Prince*, typhaneoside (**38**, 2.22–4.84 mg/g). The dominance of **34** was found earlier in cultivars of *C. officinalis* from Italy [12]; **38**, from Serbia [13].

TABLE 6. Contents of Anthocyanins in Tubular (T) and Peripheral (P) Florets of *C. officinalis* Varieties, mg/g[a]

Compound,[b] compound group	*Egypt Sun*		*Flame Dancer*		*Indian Prince*		*Red Black Centered*	
	T	P	T	P	T	P	T	P
Cy-3-*O*-Glc	2.41	0.97	3.01	1.24	0.85	Tr.	1.57	1.15
Cy-3,5-*O*-Glc	0.52	Tr.	0.83	Tr.	–	–	0.44	Tr.
Cy-3-*O*-Rut	1.05	0.30	1.49	0.56	–	–	0.89	0.64
Dp-3-*O*-Glc	0.89	–	0.55	Tr.	–	–	0.41	–
Mv-3-*O*-Glc	0.65	0.38	0.56	0.20	0.39	–	0.21	0.16
Pn-3-*O*-Glc	0.61	Tr.	0.89	Tr.	0.35	–	0.28	Tr.
Pg-3,5-*O*-Glc	1.12	Tr.	1.31	0.39	–	–	0.64	0.48
Pt-3-*O*-Glc	1.59	0.41	2.32	0.63	–	–	0.99	0.83
Identified	8.84	2.06	10.96	3.02	1.59	Tr.	5.43	3.26
Total content[c]	9.45	3.57	12.61	3.92	2.35	Tr.	6.24	3.47

[a]Of air-dried raw material mass; [b]Cy, cyanidine; Dp, delphinidin; Mv, malvidin; Pn, peonidin; Pg, pelargonidin; Pt, petunidin; [c]spectrophotometric method. Tr.: traces.

Anthocyanins (0.79–3.12 mg/g) were found in four varieties of *C. officinalis* (*Egypt Sun*, *Flame Dancer*, *Indian Prince*, and *Red Black Centered*). A distinguishing feature of these varieties was intense raspberry-red pigmentation in the central flower part. Chromatographic (HPLC) separation showed the presence of eight compounds that were identified as 3-*O*-glucopyranosides of cyanidine, delphinidin, malvidin, peonidin, pelargonidin, and petunidin; 3,5-di-*O*-glucopyranosides of cyanidine and pelargonidin; and cyanidine-3-*O*-rutinoside (Table 6). The dominant compound was cyanidine-3-*O*-glucopyranoside, the contents of which in tubular and peripheral florets were 0.85–3.01 and 0.97–1.24 mg/g, respectively. Tubular florets were the main location of this compound group with the greatest (12.61 mg/g) concentration found for variety *Flame Dancer*. Anthocyanins were found in *C. officinalis* for the first time.

EXPERIMENTAL

Varieties *Big Orange*, *Egypt Sun*, *Flame Dancer*, *Geisha Girl*, *Indian Prince*, *Radio*, and *Red Black Centered* were grown in the open at experimental plantations of the IGEB, SB, RAS (2011) from authenticated seeds obtained at the N. V. Tsitsin Main Botanical Garden, RAS (Moscow, Russia). The agrotechnical conditions recommended for *C. officinalis* were used for cultivation [14]. Florets and morphological groups were collected during full flowering (June-July); seeds and roots, during fruiting (August). Samples of raw materials are preserved in the Herbarium of the IGEB, SB, RAS (No. FAs/to-15/04-02/0811i, FAs/to-15/05-02/0811i, FAs/to-15/06-02/0811i, FAs/to-15/07-02/0811i, FAs/to-15/08-02/0811i, FAs-to-15/09-02/0811i, and FAs/to-15/10-02/0811i).

Column chromatography (CC) used silica gel (SiO_2, Sigma), reversed-phase silica gel (RP-SiO_2, Sigma), Sephadex LH-20 (Pharmacia), polyamide (Woelm), and Amberlite XAD7HP (Sigma). Spectrophotometric studies were performed on an SF-2000 spectrophotometer (OKB Spektr); MS analysis, on an MAT 8200 high-resolution mass spectrometer (Finnigan). IR spectra were recorded in KBr pellets (1:100) on an FT-801 IR-Fourier spectrometer (HPF Simeks); NMR spectra, on a VXR 500S NMR spectrometer (Varian). Total contents of flavonoids were determined by differential spectrophotometry calculated as narcissin [15]; carotenoids and anthocyanins, by spectrophotometry calculated as β-carotene and cyanidine-3-*O*-glucopyranoside, respectively [16]; essential oil, gravimetrically after steam distillation; water-soluble polysaccharides, by the anthrone–H_2SO_4 method [17]. Antiradical activity with respect to the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined by the Seyoum method [18]; peroxide degradation of β-carotene (CBA), in the DMSO–H_2O_2–linoleic acid system [19].

Extraction and Fractionation. Ground florets of *C. officinalis* variety *Big Orange* (1.4 kg) were extracted successively by 96, 70, and 50% EtOH (×3, 1:20, 40°C). The combined extracts were concentrated to an aqueous residue and extracted completely with hexane, $CHCl_3$, EtOAc, and BuOH to afford five fractions (mass, yield, % of air-dried raw material mass): hexane (F-1, 72.66 g, 5.19%), $CHCl_3$ (F-2, 57.68 g, 4.12%), EtOAc (F-3, 14.70 g, 1.05%), BuOH (F-4, 63.14 g, 4.51%), and H_2O residue (F-5, 60.90 g, 4.35%). Fraction F-2 (25 g) was separated using CC over SiO_2 (3 × 90 cm, C_6H_{14}–EtOAc eluent, 100:0→70:30) with subsequent rechromatography over RP-SiO_2 (2 × 50 cm, H_2O–MeCN eluent, 100:0→0:100). A total of

nine compounds were identified as vanillic (14 mg, **3**), syringic (18 mg, **4**), cinnamic (24 mg, **5**) [20], *p*-coumaric (11 mg, **6**), *o*-coumaric (5 mg, **7**) [21], ferulic (14 mg, **8**), and isoferulic acids (8 mg, **9**) [22]; quercetin (9 mg, **10**), and isorhamnetin (16 mg, **11**) [23]. Fractions F-3 (12 g) and F-4 (60 g) were separated by CC over polyamide (4 × 100 cm, H_2O–EtOH eluent, 100:0→96:4), SiO_2 (3 × 60 cm, C_6H_{14}–EtOAc eluent, 100:0→70:30; EtOAc–EtOH, 100:0→70:30), Sephadex LH-20 (3 × 70 cm, EtOH–H_2O eluent, 96:4→0:100), RP-SiO_2 (2 × 50 cm, H_2O–MeCN eluent, 100:0→0:100), and preparative TLC on SiO_2 (toluene–EtOAc–HCOOH, 5:4:1). This resulted in the isolation of 29 compounds including from fraction F-3 isorhamnetin-3-*O*-(2″-acetyl-)-*β*-D-glucopyranoside (18 mg, **1**), isorhamnetin-3-*O*-(2″,6″-diacetyl-)-*β*-D-glucopyranoside (12 mg, **2**); caffeic acid (27 mg, **12**) [21]; 3-*O*- (68 mg, **13**), 5-*O*- (24 mg, **14**), 1,3-di-*O*- (11 mg, **15**), 3,4-di-*O*- (18 mg, **16**), 3,5-di-*O*- (33 mg, **17**), 4,5-di-*O*- (14 mg, **18**), 1,3,5-tri-*O*- (20 mg, **19**), and 3,4,5-tri-*O*-caffeylquinic acids (7 mg, **20**); 5-*O*-ferulylquinic acid (5 mg, **21**) [24], quercitrin (18 mg, **22**), isoquercitrin (35 mg, **23**) [25], quercetin-3-*O*-(2″-acetyl-)-*β*-D-glucopyranoside (12 mg, **24**), quercetin-3-*O*-(6″-acetyl)-*β*-D-glucopyranoside (14 mg, **25**), quercetin-3-*O*-(2″,6″-diacetyl)-*β*-D-glucopyranoside (4 mg, **26**) [26], isorhamnetin-3-*O*-*β*-D-glucopyranoside (31 mg, **27**) [25], isorhamnetin-3-*O*-*α*-L-rhamnopyranoside (5 mg, **28**), calendoflaside [isorhamnetin-3-*O*-(2″-*α*-L-rhamnopyranosyl)-*α*-L-rhamnopyranoside] (37 mg, **29**) [9], and isorhamnetin-3-*O*-(6″-acetyl)-*β*-D-glucopyranoside (16 mg, **30**) [6]. Fraction F-4 afforded 4-*O*-caffeylquinic acid (12 mg, **31**) [24], quercetin-3-*O*-(2″-*α*-L-rhamnopyranosyl)-*α*-L-rhamnopyranoside (32 mg, **32**) [10], rutin (212 mg, **33**), narcissin [isorhamnetin-3-*O*-(6″-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside] (3.25 g, **34**) [25], calendoflavoside [isorhamnetin-3-*O*-(2″-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside] (52 mg, **35**), calendoflavobioside [quercetin-3-*O*-(2″-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside] (107 mg, **36**) [9], manghaslin [quercetin-3-*O*-(2″,6″-di-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside] (114 mg, **37**) [27], and typhaneoside [isorhamnetin-3-*O*-(2″,6″-di-*α*-L-rhamnopyranosyl)-*β*-D-glucopyranoside] (2.28 g, **38**) [10]. Fraction F-5 (50 g) was placed on Amberlite XAD7HP (300 g) and eluted with H_2O and EtOH (50%). The EtOH eluate was concentrated and chromatographed over RP-SiO_2 (2 × 50 cm, H_2O–MeCN eluent, 100:0→0:100) to isolate the three compounds 1-*O*-caffeylglucose (14 mg, **39**), 1-*O*-caffeylquinic acid (21 mg, **40**), and 3-*O*-*p*-coumarylquinic acid (9 mg, **41**) [24].

Isorhamnetin-3-*O*-(2″-acetyl-)-*β*-D-glucopyranoside (1). $C_{24}H_{24}O_{13}$. HR-ESI-MS *m*/*z*: 543.421 ($[M + Na]^+$; calcd 543.441). + FAB-MS *m*/*z*: 521 $[M + H]^+$, 317 $[(M – Glc – acetate) + H]^+$. UV spectrum (MeOH, λ_{max}, nm): 256, 266 sh, 359; + $AlCl_3$ 270, 306, 405; + $AlCl_3$/HCl 270, 305, 406; + NaOAc 275, 321, 389; + NaOAc/H_3BO_3 257, 266 sh, 360; + NaOMe 272, 332, 418. IR spectrum (ν_{max}, cm^{-1}): 1725, 1652 (ν_{CO}). ^1H NMR (500 MHz, MeOH-d_4, δ, ppm) and ^{13}C NMR (125 MHz, MeOH-d_4, δ, ppm), see Table 2.

Isorhamnetin-3-*O*-(2″,6″-diacetyl-)-*β*-D-glucopyranoside (2). $C_{26}H_{26}O_{14}$. HR-ESI-MS *m*/*z*: 585.263 ($[M + Na]^+$; calcd 585.479). + FAB-MS *m*/*z*: 563 $[M + H]^+$, 317 $[\{M – Glc – (2 \times acetate)\} + H]^+$. UV spectrum (MeOH, λ_{max}, nm): 256, 265 sh, 359; + $AlCl_3$ 270, 305, 405; + $AlCl_3$/HCl 271, 305, 406; + NaOAc 275, 322, 390; + NaOAc/H_3BO_3 257, 266 sh, 360; + NaOMe 272, 332, 419. IR spectrum (ν_{max}, cm^{-1}): 1724, 1652 (ν_{CO}). ^1H NMR and ^{13}C NMR, see Table 2.

Isorhamnetin-3-*O*-(6″-acetyl-)-*β*-D-glucopyranoside (30). $C_{27}H_{30}O_{16}$. HR-ESI-MS *m*/*z*: 543.421 ($[M + Na]^+$; calcd 543.441). + FAB-MS *m*/*z*: 521 $[M + H]^+$, 317 $[(M – Glc – acetate) + H]^+$. UV spectrum (MeOH, λ_{max}, nm): 255, 266 sh, 360; + $AlCl_3$ 270, 306, 405; + $AlCl_3$/HCl 270, 304, 406; + NaOAc 275, 321, 389; + NaOAc/H_3BO_3 257, 266 sh, 361; + NaOMe 272, 332, 420. IR spectrum (ν_{max}, cm^{-1}): 1722, 1650 (ν_{CO}). ^1H NMR (500 MHz, MeOH-d_4, δ, ppm, J/Hz): 1.64 (3H, s, Glc 6″-COC$\underline{H}_3$), 3.11–3.36 (4H, m, Glc H-2″-5″), 3.81 (3H, s, 3′-OC$\underline{H}_3$), 4.05 (1H, m, Glc H-6″$_B$), 4.21 (1H, dd, J = 2.0, 12.0, Glc H-6″$_A$), 5.81 (1H, d, J = 7.7, Glc H-1″), 6.14 (1H, d, J = 2.2, H-6), 6.33 (1H, d, J = 2.2, H-8), 6.89 (1H, d, J = 9.0, H-5′), 7.44 (1H, dd, J = 9.0, 1.8, H-6′), 7.85 (1H, d, J = 2.0, H-2′). ^{13}C NMR (125 MHz, MeOH-d_4, δ, ppm): 20.1 (C, 6″-OC$\underline{C}H_3$), 55.1 (CH_3, 3′-O$\underline{C}H_3$), 63.7 (CH_2, Glc C-6″), 70.1 (CH, Glc C-4″), 73.3 (CH, Glc C-2″), 76.6 (CH, Glc C-5″), 77.7 (CH, Glc C-3″), 93.4 (CH, C-8), 100.0 (CH, C-6), 101.4 (CH, Glc C-1″), 104.2 (C, C-10), 112.7 (CH, C-2′), 115.8 (CH, C-5′), 121.5 (C, C-1′), 122.6 (CH, C-6′), 133.8 (C, C-3), 146.3 (C, C-4′), 149.1 (C, C-3′), 156.2 (C, C-9), 156.3 (C, C-2), 161.1 (C, C-5), 164.3 (C, C-7), 170.9 (CH_3, 6″-O$\underline{C}CH_3$), 178.2 (C, C-4).

Total Hydrolysis of 1 and 2. The compound (2 mg) was dissolved in TFA (5 mL, 5%) in Me_2CO and heated at 100°C (2 h). The hydrolysate was concentrated in vacuo, dissolved in MeOH, and analyzed by HPLC. The presence of isorhamnetin (t_R 11.21 min, conditions 1) and glucose (t_R 14.14 min, conditions 2) was established.

Alkaline Hydrolysis of 1 and 2. The compound (2 mg) was dissolved in NaOH solution (1 mL, 0.4%) and heated at 50°C (30 min). The hydrolysate was neutralized with HCl (0.4%) and placed on a RP-SiO_2 Diapak C16M cartridge (BioKhimMak) that was eluted with H_2O and EtOH (70%). Analysis of the resulting eluates by HPLC found isorhamnetin-3-*O*-*β*-D-glucopyranoside in the EtOH eluate (t_R 6.98 min, conditions 1) and acetate in the H_2O eluate (t_R 18.24 min, conditions 3).

Hydrolysis of 1 and 2 by β-Glucosidase. The compound (3 mg) and β-glucosidase from *Prunus amygdalus* (50 U, Sigma) were suspended in phosphate buffer (Na_2HPO_4–NaH_2PO_4, pH 7.3), incubated at 37°C (12 h), heated at 100°C (24 h), and centrifuged (6,000 g). The supernatant was extracted with EtOAc (2 × 3 mL), concentrated in vacuo, and dissolved in MeOH. HPLC detected isorhamnetin (t_R 11.21 min, conditions 1).

HPLC. We used a Milikhrom A-02 microcolumn liquid chromatograph (EkoNova). Conditions 1: Pronto SIL-120-5-C18 AQ column (2 × 75 mm, ∅ 5 μm; Metrohm AG); mobile phase: $LiClO_4$ (0.2 M) in $HClO_4$ (0.003 M) (A), MeCN (B); gradient mode (%B): linear gradient 0–20 min 5–100%; ν 200 μL/min; column temperature 40°C; λ 360 nm. Conditions 2: Separon 5-NH_2 column (2 × 75 mm, ∅ 5 μm; Tessek Ltd.); mobile phase: 75% MeCN; isocratic mode (0–20 min); ν 100 μL/min; column temperature 35°C; λ 190 nm. Conditions 3: Rezex ROA-Organic Acid column (2 × 75 mm, ∅ 5 μm; Phenomenex); mobile phase: H_2SO_4 (0.005 M); isocratic mode (0–30 min); ν 300 μL/min; column temperature 35°C; λ 210 nm. Plant raw material was analyzed quantitatively using the microcolumn HPLC-UV, for which raw material (40 mg) was placed into an Eppendorf tube (2 mL), treated with EtOH (1 mL, 60%), sonicated (50 kHz, 30 min, 40°C), and centrifuged (6,000 g, 20 min).

The resulting extract (500 μL) was diluted with H_2O (1:5) and placed onto a polyamide cartridge (1 g) that was eluted with H_2O (25 mL), EtOH (90%) (eluate 1), and NH_3 solution (0.5%) in EtOH (90%) (eluate 2). Eluates 1 and 2 were concentrated to dryness, dissolved in EtOH (1 mL, 70%), filtered through a membrane filter (0.45 μm), and used for the analysis (1 μL). Eluate 1 was used for analysis of flavonoid glycosides; eluate 2, of acylated flavonoids and phenylpropanoids. Conditions: Milikhrom A-02 microcolumn liquid chromatograph (EkoNova); Pronto SIL-120-5-C18 AQ column (2 × 75 mm, ∅ 5 μm; Metrohm AG); mobile phase: $LiClO_4$ (0.2 M) in $HClO_4$ (0.006 M) (A), MeCN (B); gradient mode (%B): 0–7.5 min 11–18%, 7.5–13.5 min 18%, 13.5–15 min 18–20%, 15–18 min 20–25%, 18–24 min 25%, 24–30 min 25–100%; ν 100 μL/min; column temperature 35°C, λ 330 and 360 nm. The contents of pure components were calculated from calibration curves that were constructed using commercial samples of standard compounds (caffeic acid, 3-*O*-, 4-*O*-, 5-*O*-caffeylquinic acids, 1,3-di-*O*-caffeylquinic acid, rutin, isoquercitrin, narcissin, isorhamnetin-3-*O*-β-D-glucopyranoside, all SigmaAldrich), isolated samples of compounds with purity ≥95% (manghaslin, calendoflavobioside, typhaneoside, calendoflavoside, calendoflaside), and external reference samples [3,5- and 4,5-di-*O*-caffeylquinic acids using 1,3-di-*O*-caffeylquinic acid; quercetin-3-*O*-(2″-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside using rutin; quercetin-3-*O*-(6″-acetyl-)-β-D-glucopyranoside using isoquercitrin; isorhamnetin-3-*O*-(6″-acetyl-)-β-D-glucopyranoside using isorhamnetin-3-*O*-β-D-glucopyranoside]. Anthocyanins were analyzed on a Summit HPLC (Dionex); Diasfer-110-C18 column (4 × 150 mm, ∅ 5 μm; BioKhimMak); mobile phase: MeCN–HCOOH–H_2O (4:5:41); isocratic mode (20 min); ν 1 mL/min; column temperature 30°C, λ 530 nm.

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