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Anti-inflammatory Potential of Flavonoids from the Aerial Parts of *Corispermum marschallii*

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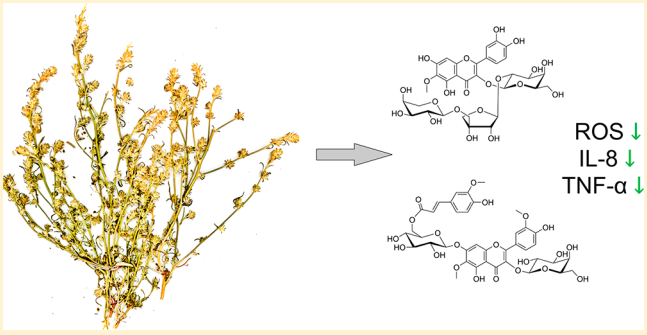
Supporting Information

ABSTRACT: The isolation of phenolics from aerial parts of *Corispermum marschallii* yielded a total of 13 compounds including nine previously undescribed patuletin and spinacetin glycosides. These were identified as patuletin 3-*O*-*β*-D-galactopyranosyl-7-*O*-*β*-D-glucopyranoside (**1**), spinacetin 3-*O*-*β*-D-galactopyranosyl-7-*O*-*β*-D-glucopyranoside (**2**), patuletin 3-*O*-(6″-*O*-*β*-D-glucopyranosyl)-*β*-D-galactopyranoside (**3**), patuletin 3-*O*-(6″-*O*-*α*-L-arabinopyranosyl)-*β*-D-galactopyranoside (**4**), patuletin 3-*O*-(2″-*O*-(5‴-*O*-*α*-L-arabinopyranosyl)-*β*-D-apiofuranosyl)-*β*-D-galactopyranoside (**5**), patuletin 3-*O*-(2″-*O*-*β*-D-apiofuranosyl)-*β*-D-galactopyranoside (**6**), spinacetin 3-*O*-*β*-D-galactopyranoside (**7**), patuletin 3-*O*-*β*-D-galactopyranosyl-7-*O*-(6‴-*O*-feruloyl)-*β*-D-glucopyranoside (**8**), and spinacetin 3-*O*-*β*-D-galactopyranosyl-7-*O*-(6‴-*O*-feruloyl)-*β*-D-glucopyranoside (**9**). Structure elucidation was based on UV−visible, multistage MS, and 1D and 2D NMR spectroscopy and chemical derivatization, which allowed the identification on the glycosides with two different hexose moieties occurring at different positions of the aglycones. Most of the compounds tested inhibited the production of pro-inflammatory factors such as ROS, IL-8, and TNF-*α* in stimulated neutrophils.



Corispermum marshallii Steven (syn. *C. borysthenicum* Andrz.[1]), commonly known as Marschall's bugseed or gray bugseed (from the German, *Marschall Wanzensame* or *Grauer Wanzensame*), belongs to the family Amaranthaceae. It is an annual herbaceous plant that grows 20−50 cm high with an arborescent stem and oblong-lanceolate leaves and spikes. The plant is found mainly in Central and Eastern Europe in gravelly or sand habitats.[2]

Available research on the phytochemical composition of *Corispermum* species has shown that species belonging to this genus contain isoquinoline alkaloids such as salsoline and salsolidine. In particular, the presence of salsolidine in aerial parts of *C. marschallii* was confirmed.[3] The phytochemical analysis of *C. leptopyrum*[4] revealed another unknown basic compound as well. Other research has revealed that seeds of *Corispermum* species contain small amounts of ecdysteroids.[5] Also, a previous study has indicated the presence of vitamins and flavonoid glycosides in *C. papillosum* collected in a desert region of Central Asia,[6] revealing that studying the phenolic profiles of other *Corispermum* species would be a worthwhile endeavor.

From previous studies, it is unclear whether or not *Corispermum* species might be useful in pharmacotherapy. Although there are some reports that various Asian species of *Corispermum* were used in traditional Chinese medicines for treating a wide variety of maladies,[7−10] none of their effects can be attributed to the activity of alkaloids. These activities may suggest possible anti-inflammatory effects; thus, further exploration of the content of phenols and their antioxidant and anti-inflammatory activities may be justified.

The aim of the present study was to develop a UHPLC-DAD-MS method to analyze the phenolic profile of the extracts from aerial parts of *C. marschallii* and to isolate and identify the major flavonoids present in this plant material. Additionally, the bioactivities of the isolated compounds were evaluated using a human neutrophil model.

RESULTS AND DISCUSSION

The structures of 13 compounds isolated from *C. marschallii* were determined based on UV−vis spectroscopy, multistage MS (Table S1, Supporting Information), and NMR spectroscopy (Figure 1), and a chromatographic method was developed to identify the monosaccharide units present and determine their configurations on hydrolysis. ^{1}H NMR spectra

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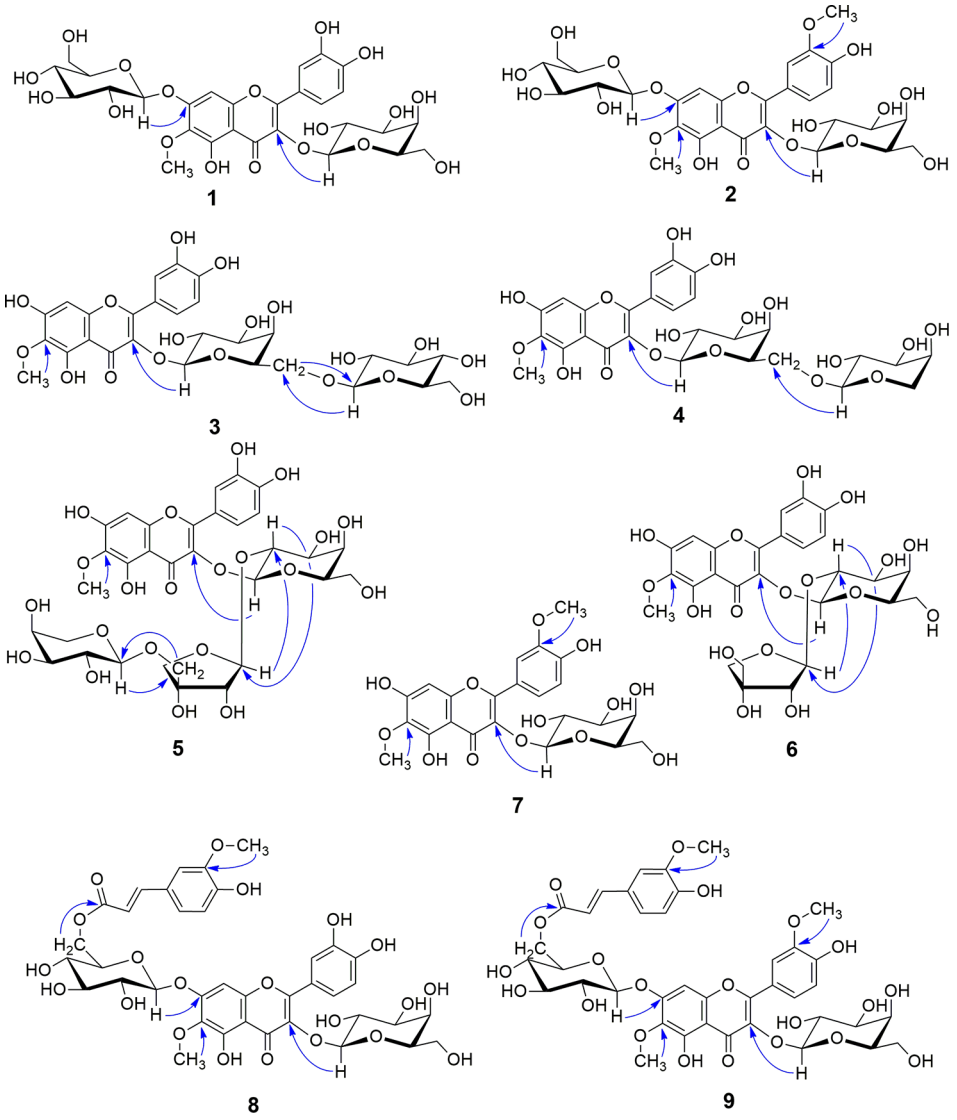

Figure 1. Relevant HMBC correlations occurring in spectra of compounds isolated from *C. marschallii*.

were recorded for each isolated compound and compared with the available literature. For structure elucidation of the majority of the new isolated compounds, further NMR experiments, such as ^{13}C NMR, HSQC, HMBC, and COSY, were performed.

The aglycone in compound **1** was identified by analysis of the chemical shifts in the ^{13}C NMR spectrum and the HMBC correlations proving the linkage of the methoxy substituent (represented by a high-intensity singlet at δ_H 3.76 ppm) to C-6 carbon (δ_C 132.2 ppm). After comparison with previously published data,[11] the aglycone was identified unequivocally as patuletin. The chemical shifts of the glycosyl signals were assigned based on the ^{13}C NMR and HSQC experiments and previously published data on D-galactopyranose[12,13] and D-glucopyranose moieties.[14] The glycosylation site of the D-galactosyl unit was determined based on an HMBC experiment, which showed a cross-peak between the H-1″ doublet (δ_H 5.41, J = 7.7 Hz) and the C-3 carbon (δ_C 133.4 ppm). Moreover, the value of the coupling constant indicated that the glycoside was the β-anomer. The D-glucosyl moiety was linked to the aglycone at the C-7 position based on an HMBC experiment, which showed a cross-peak between the H-1‴ doublet (δ_H 5.14, J = 7.5 Hz) and the C-7 carbon (δ_C 156.4 ppm). Thus, compound **1** was determined as patuletin 3-*O*-β-D-galactopyranosyl-7-*O*-β-D-glucopyranoside, a new compound. Earlier reports of patuletin glycosides identified in plants from the Amaranthaceae family (particularly those formerly classified as in the family Chenopodiaceae) have concerned only 3-*O*-glucosides, and not 3-*O*-galactosides.[15,16]

The NMR data of compound **2** were found to be similar to those of compound **1**. The chemical shifts and correlations in the 2D spectra were mostly comparable; the exception was the presence of an additional singlet in the ^{1}H NMR spectrum (δ_H 3.85 ppm) and a ^{13}C NMR signal (δ_C 56.0 ppm) caused by the presence of a second methoxy group. The position of this substituent was determined from the HMBC experiment in which the singlet was correlated with the C-3′ carbon in the B ring of flavonol (δ_C 147.0 ppm). The NMR data of the aglycone were in agreement with previous reports on spinacetin 3-*O*-glycoside.[11] Compound **2** was found to be a dihexoside based on its MS, which showed neutral losses characteristic of the cleavage of a hexoside (m/z 162 amu). The NMR data allowed the assignment of **2** as spinacetin 3-*O*-β-D-galactopyranosyl-7-*O*-β-D-glucopyranoside with the chemical shifts and

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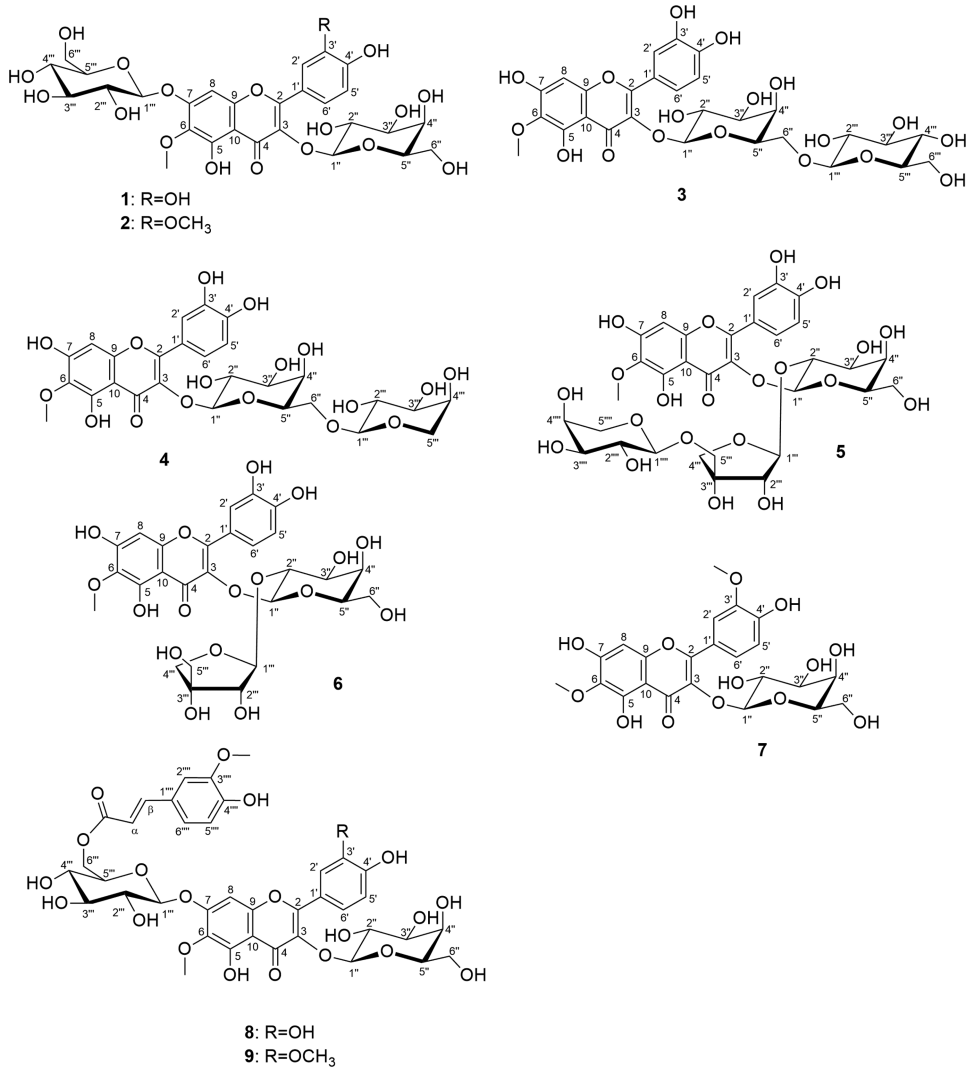
Chart 1



coupling constants of the spectra recorded in DMSO-d_6 being analogous to those in the same spectra of compound **1**.

Compound **3** gave the same aglycone and glycosyl moieties as were observed for compound **1**. Changes in the chemical shift of the H-1‴ doublet (δ = 4.06 ppm) and the shifts of H-5″ and H-6″ along with the changes in the HMBC spectrum (i.e., the absence of the H-1‴/C-7 cross-peak and the appearance of two new correlations at H-1‴/C-6″ and vice versa) led to the conclusion that compound **3** is patuletin 3-*O*-(6″-*O*-*β*-D-glucopyranosyl)-*β*-D-galactopyranoside. Compound **3** has not been reported previously.

The presence of a pentosyl unit in the structure of compound **4** was suspected based on the MS fragmentation analysis and was confirmed by the ^{13}C NMR data. Both the ^{1}H and ^{13}C NMR chemical shifts and the coupling constant of the anomeric proton doublet (δ = 3.97 ppm, J = 6.7 Hz, 1H) were consistent with the published spectra of *α*-L-arabinopyranosides recorded in DMSO-d_6.[17,18] The HMBC correlation between the H-1‴ doublet and C-6″ was indicative of linear glycosylation and a structure analogous to that of compound **3**. Compound **4** was thus established as patuletin 3-*O*-(6″-*O*-*α*-L-arabinopyranosyl)-*β*-D-galactopyranoside, a new natural compound.

The MS fragmentation pattern and the NMR spectra of compound **5** were more complex and showed signals from both a hexose and two pentose moieties. The results of the hydrolysis experiments indicated the presence of D-galactose, L-arabinose, and D-apiose moieties. The galactopyranose structure was confirmed as described above for compound **1**, and the signals from the arabinopyranose atoms were compared with the spectra of arabinopyranosides recorded in CD_3OD.[19,20] An apiofuranoside unit was identified based on the DEPT-edited HSQC data, in which inverted signals were observed for two groups of cross-peaks, revealing the presence of two CH_2 units. Additionally, the ^{13}C NMR signal corresponding to the C-3‴ carbon (δ = 80.0 ppm) did not correlate to any ^{1}H NMR signals in the HSQC spectrum, meaning it was tertiary. Chemical shifts were assigned based on the HMBC and COSY spectra and comparison with available data,[21,22] which enabled the elucidation of an apiofuranose unit. The coupling constant of the anomeric proton doublet (δ_H 5.48 ppm, J = 0.8 Hz) indicated a *trans* relationship between the H-1‴ and H-2‴ protons, which in the case of the apiofuranose unit corresponded to a *β*-configuration.[21,22] The HMBC cross-peaks between the H-1‴ doublet of the arabinofuranosyl moiety and the C-2″ carbon of the galactopyranosyl unit (and, inversely, the H-2″/C-1‴ cross-peak) indicated the glycosylation position of the first pentoside, a *β*-apiofuranoside moiety, whereas analysis of the cross-peak between the H-1⁗ doublet of

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the arabinopyranosyl moiety and the signal from the C-5‴ carbon (as well as the H-5‴/C-1⁗ cross-peak) indicated the second residue to be a pentoside. Thus, the structure of the new compound **5** was determined as patuletin 3-*O*-(2″-*O*-(5‴-*O*-α-L-arabinopyranosyl)-β-D-apiofuranosyl)-β-D-galactopyranoside.

Compound **6**, a further new compound, was identified by analogy to **5**. The structure of **6** lacked only the arabinopyranoside moiety, which resulted in slightly different chemical shifts for the H-5‴ and C-5‴ signals. Thus, the structure of **6** was elucidated as patuletin 3-*O*-(2″-*O*-β-D-apiofuranosyl)-β-D-galactopyranoside.

Compound **7** was found to be a monohexoside based on its MS data. The NMR spectra of compound **7** were found to be similar to those of compound **2**. The chemical shifts and correlations in the 2D spectra were comparable in the case of the aglycone, with the difference concerning the lack of the glucosyl moiety that was present in the structure of compound **2**. The galactosyl unit in compound **7** was identified by analogy to compound **1**, which confirmed that **7** is spinacetin 3-*O*-β-D-galactopyranoside, a new substance.

Compounds **8** and **9** were determined to be derivatives of compounds **1** and **2**, respectively, based on their NMR spectra, which showed comparable chemical shifts and the same 2D correlations between the analogous signals. The resonances in the NMR spectra of compounds **8** and **9** that were noted in the spectra of **1** and **2** were signals for an additional moiety including two doublets and a doublet of doublets (with chemical shifts that indicated an additional aromatic ring with an ABX coupling pattern) and doublets with large coupling constants ($J \approx 15.9$ Hz) that corresponded to olefinic protons and another high-intensity singlet from a methoxy group. The latter showed an HMBC correlation with the C-3⁗ carbon in the aromatic system described. The ^{13}C NMR data included a second signal from a carbonyl group (δ_C 166.7 ppm) and signals that showed an HMBC cross-peak with the H-6‴ glucopyranosyl proton. These data allowed the identification of a feruloyl moiety, and thus **8** and **9** were assigned as patuletin 3-*O*-β-D-galactopyranosyl-7-*O*-(6‴-*O*-feruloyl)-β-D-glucopyranoside and spinacetin 3-*O*-β-D-galactopyranosyl-7-*O*-(6‴-*O*-feruloyl)-β-D-glucopyranoside, respectively. Similar compounds have been isolated from *Atriplex littoralis* (Amaranthaceae)[15] and *Chenopodium bonus-henricus* (Amaranthaceae).[23] Nevertheless, both **8** and **9** are new.

The potential anti-inflammatory activities of the isolated compounds were evaluated using a human neutrophil model. None of the compounds were cytotoxic to the cells used up to 50 μM based on cytometric experiments (data not shown). The ability of each test compound to decrease the production or release of pro-inflammatory factors such as reactive oxygen species (ROS), interleukin 8 (IL-8), and tumor necrosis factor-α (TNF-α) was evaluated. As expected from previous reports,[24−27] all constituents were able to decrease the production of ROS from f-MLP (*N*-formylmethionyl-leucyl-phenylalanine)-stimulated neutrophils (Figure S77, Supporting Information). Compounds were tested in the concentration range of 5 to 20 μM. The activities of most of the tested compounds were concentration-dependent, and their IC_{50} values are shown in Table 3. The statistically significant differences between the obtained values were analyzed. The most potent activities [comparable to the positive control, quercetin (IC_{50} 4.4 ± 0.3 μM)] were observed for compounds **3**−**5**, **9**, and patuletin 3-*O*-β-D-galactopyranoside.

Each of the isolated compounds was tested for the ability to decrease the production of IL-8 and TNF-α from lipopolysaccharide (LPS)-stimulated neutrophils at the concentration range 10 to 50 μM. Patuletin 3-*O*-β-D-galactopyranoside was confirmed to be active toward the inhibition of TNF-α production. The most potent activities were observed for compounds **3**, **5**, **6**, patuletin 3-*O*-β-D-galactopyranoside, and 6-methoxykaempferol 3-*O*-β-D-galactopyranoside, and the IC_{50} values ranged from 7.7 ± 1.5 μM for patuletin 3-*O*-β-D-galactopyranoside to 12.8 ± 4.5 μM for compound **6** (Table 3). No obvious relationship between the chemical structure of the tested flavonoid and their potential anti-inflammatory activities could be established based on the results obtained. It was confirmed that all the investigated compounds can be considered, to some extent, potential anti-inflammatory agents. Whenever the activity of natural products is considered, the question about their bioavailability must be addressed. In general flavonoids exhibit a very low bioavailability after oral intake.[28] However, several studies have shown that the uptake of flavonoids aglycones and/or glycosides can occur at different levels of the gastrointestinal tract, leading to their presence in the circulation in unchanged form.[29] Additionally, it was proven that administration of formulations containing multiple plant components (similar to those in which other *Corispermum* species can be found in TCM) may increase the absorption of flavonoids after oral intake.[30]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a PerkinElmer polarimeter model 241 using a 1 cm microcell. NMR spectra were acquired using Varian VNMRS Oxford 300 MHz (for compounds **2**−**7** and all known compounds) and Bruker AVANCE II HD 500 MHz spectrometers (for compounds **1**, **8**, and **9**). High-resolution mass spectra were acquired using a time-of-flight high-resolution mass 6230 mass spectrometer (Agilent). The parameters for the ESI source were as follows: nebulizer pressure 45 psi; dry gas flow 12 L/min; dry temperature 300 °C; and capillary voltage 4.5 kV. Analysis was carried out using scans from *m*/*z* 100 to 1100. Mass spectra were recorded in the positive- and negative-ion modes. UHPLC-DAD-MS analysis was conducted using a Dionex Ultimate 3000RS system coupled with an Amazon SL ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The ion trap AmazonSL mass spectrometer was equipped with an ESI interface. The eluate was introduced into the ESI interface of the mass spectrometer without splitting. The parameters for the ESI source were as follows: nebulizer pressure 40 psi; dry gas flow 9 L/min; dry temperature 300 °C; and capillary voltage 4.5 kV. Analysis was carried out using scanning from *m*/*z* 70 to 2200. Mass spectra were recorded in the positive- and negative-ion modes. A sample of the crude plant extract dissolved in methanol (20 mg/mL) was filtered through a 0.45 μm syringe filter and subjected to UHPLC-DAD-MS analysis. The separation was carried out on a Kinetex XB-C_{18} (150 mm × 3.0 mm × 2.6 μm, Phenomenex, Torrance, CA, USA) column maintained at 25 °C. The mobile phases were 0.1% HCOOH in water (A) and 0.1% HCOOH in acetonitrile (B), and elution was conducted with the following gradient: 0 min, 0% B; 60 min, 26% B; and 70 min, 95% B. The flow rate was 0.4 mL/min, and the injection volume was 2 μL of the prepared extract. The UV−vis spectra of the detected compounds were recorded over the 190−450 nm range. The chromatogram was recorded at 254 nm. Compounds were characterized based on the maxima observed in their UV−vis spectra and on their MS spectra. Acetonitrile, methanol, and formic acid for UHPLC were purchased from Merck (Darmstadt, Germany). Water for UHPLC was purified with a Millipore Simplicity System (Bedford, MA, USA). Solvents for the extraction of plant material and isolation were of analytical grade. Column chromatography was carried out on Sephadex LH-20 (POCh, Gliwice, Poland), Diaion HP-20 (Sigma−Aldrich, Steinheim, Germany),

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Table 1. NMR Spectroscopic Data for Patuletin (1, 3, 8) and Spinacetin Glycosides (2, 7, 9)

	1[a]		2[b]		3[a]		7[a]		8[a]		9[a]	
position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
flavonol moiety												
2		157.0, C		159.3, C		156.5, C		156.3, C		156.9, C		156.9, C
3		133.4, C		135.3, C		133.2, C		132.8, C		133.5, C		133.1, C
4		177.8, C		179.8, C		177.7, C		177.6, C		177.8, C		177.8, C
5		151.2, C		153.7,[d] C		151.6, C		152.4, C		151.2, C		151.3, C
6		132.2, C		133.8, C		131.3, C		131.3, C		132.3, C		132.4, C
7		156.4, C		157.8, C		157.5, C		157.7, C		156.4, C		156.4, C
8	6.93, s	93.9, CH	6.88, s	95.5, CH	6.50, s	93.9, CH	6.55, s	94.0, CH	6.98, s	93.9, CH	7.01, s	94.0, CH
9		152.1, C		153.3,[d] C		152.4, C		151.6, C		152.2, C		152.2, C
10		106.0, C		108.0, C		104.3, C		104.3, C		106.1, C		106.2, C
H_3CO-6	3.76, s	60.2, CH_3	3.91, s	61.5, CH_3	3.75, s	60.0, CH_3	3.75, s	60.0, CH_3	3.76, s	60.4, CH_3	3.76, s	60.4, CH_3
1′		121.1, C		122.8, C		121.1, C		121.1, C		121.0, C		120.9, C
2′	7.54, d (2.2)	116.2, CH	8.05, d (1.8)	114.6, CH	7.52, d (2.1)	116.0, CH	8.02, d (2.0)	113.6, CH	7.58, d (2.2)	116.2, CH	8.03, d (2.1)	113.6, CH
3′		144.9, C		148.4, C		144.8, C		147.0, C		144.9, C		147.0, C
4′		148.6, C		151.0, C		148.5, C		149.4, C		148.7, C		149.3, C
5′	6.83, d (8.5)	115.1, CH	6.92, d (8.4)	115.9, CH	6.81, d (8.5)	115.2, CH	6.92, d (8.5)	115.2, CH	6.81, d (8.5)	115.1, CH	6.88, d (8.4)	115.1, CH
6′	7.66, dd (8.5, 2.2)	122.0, CH	7.62, dd (8.4, 1.8)	123.8, CH	7.66, dd (8.5, 2.1)	122.0, CH	7.49, dd (8.4, 2.0)	121.9, CH	7.67, dd (8.5, 2.2)	122.0, CH	7.53, dd (8.4, 2.1)	121.9, CH
H_3CO-3′			3.96, s	57.0, CH_3			3.85, s	56.0, CH_3			3.85, s	56.0, CH_3
galactopyranose moiety												
1″	5.41, d (7.7)	101.5, CH	5.42, d (7.8)	104.1, CH	5.34, d (7.6)	101.8, CH	5.51, d (7.7)	71.3, CH	5.52, d (7.7)	101.5, CH	5.52, d (7.7)	101.5, CH
2″	3.57, t (8.6)	71.2, CH	3.83[c]	73.1, CH	3.53[c]	71.1, CH	3.57[c]	73.1, CH	3.58[c]	71.3, CH	3.58[c]	71.3, CH
3″	3.35[c]	73.2, CH	3.58[c]	74.9, CH	2.84, m	73.3, CH	3.41[c]	68.0, CH	3.38−3.24[c]	73.1, CH	3.38−3.24[c]	73.1, CH
4″	3.65, d (3.1)	68.0, CH	3.86[c]	70.0, CH	3.68[c]	67.9, CH	3.68, d (2.2)	76.0, CH	3.68, d (3.0)	67.9, CH	3.68, d (3.0)	67.9, CH
5″	3.33[c]	75.9, CH	3.48[c]	77.3, CH	2.93[c]	76.6, CH	3.37[c]	60.4, CH_2	3.37[c]	75.9, CH	3.37[c]	75.9, CH
6″	3.31[c]	60.2, CH_2	3.40−3.60[c]	62.2, CH_2	3.74,[c] 3.40[c]	67.1, CH_2	3.50,[c] 3.35[c]	156.3, C	3.35,[c] 3.48[c]	60.2, CH_2	3.35,[c] 3.48[c]	60.2, CH_2
glucopyranose moiety												
1‴	5.14, d (7.5)	100.0, CH	5.12, d (6.9)	101.9, CH	4.06, d (7.7)	102.9, CH			5.24, d (7.3)	100.1, CH	5.24, d (7.3)	100.1, CH
2‴	3.35[c]	73.2, CH	3.58[c]	74.8, CH	3.36[c]	73.0, CH			3.35−3.24[c]	73.1, CH	3.35−3.24[c]	73.1, CH
3‴	3.32[c]	76.7, CH	3.43[c]	77.9, CH	2.98[c]	76.6, CH			3.36[c]	76.3, CH	3.36[c]	76.3, CH
4‴	3.18[c], t (8.8)	69.6, CH	3.41[c]	71.3, CH	2.97[c]	69.9, CH			3.34[c]	69.3, CH	3.34[c]	69.3, CH
5‴	3.45[c], m	77.3, CH	3.53[c]	78.5, CH	3.55[c]	73.8, CH			3.79[c]	73.9, CH	3.79[c]	73.9, CH
6‴	3.45[c], m	60.6, CH_2	3.40−3.60[c]	62.5, CH_2	3.55[c], 3.36[c]	60.9, CH_2			4.39, dd (10.2, 1.7)	63.0, CH_2	4.39, dd (10.2, 1.7)	63.0, CH_2
									4.24, dd (12.2, 5.1)		4.24, dd (12.2, 5.1)	
feruloyl moiety												
C═O										166.7, C		166.7, C
α									6.40, d (15.9)	114.0, CH	6.40, d (15.9)	114.0, CH
β									7.48, d (15.9)	145.4, CH	7.48, d (15.9)	145.4, CH
1‴′										125.4, C		125.4, C
2‴′									7.22, d (1.9)	111.0, CH	7.22, d (1.9)	111.0, CH
3‴′										147.9, C		147.9, C
4‴′										149.6, C		149.6, C
5‴′									6.69, d (8.1)	115.5, CH	6.69, d (8.1)	115.5, CH
6‴′									6.91, dd (8.3, 1.9)	123.1, CH	6.91, dd (8.3, 1.9)	123.1, CH
H_3CO									3.74, s	55.6, CH_3	3.74, s	55.6, CH_3

[a]Recorded in DMSO-d_6. [b]Recorded in CD_3OD. [c]Signals overlapped, chemical shifts were derived from HSQC spectrum. [d]Interchangeable.

and Toyopearl HW-40F (Tosoh, Shunan, Japan). Thin-layer chromatography (TLC) was performed on Merck silica gel 60 (0.25 mm) plates.

Plant Material. The aerial parts of *C. marschallii* (700 g) were collected on the banks of the Vistula River (Masovian District, Poland; GPS: N 52°17′36.7″, E 20°58′19.4″) in September 2014, and the species was authenticated by S. Granica and C. Zidorn. A voucher specimen has been deposited in the plant collection of the Department of Pharmacognosy and Molecular Basis of Phytotherapy Medical University of Warsaw, Poland. A scan of the voucher is available in Figure S3 (Supporting Information).

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Table 2. NMR Spectroscopic Data for Patuletin Glycosides (4, 5, 6)

	4[a]		5[b]		6[b]	
position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
patuletin moiety						
2		156.5, C		158.5, C		158.3, C
3		133.1, C		134.7, C		134.6, C
4		177.7, C		179.7, C		179.8, C
5		152.4, C		153.7, C		153.8, C
6		131.2, C		132.5, C		132.5, C
7		157.4, C		158.4, C		158.4, C
8	6.51, s	93.8, CH	6.51, s	94.8, CH	6.47, s	94.7, CH
9		151.5, C		153.8, C		153.6, C
10		104.4, C		106.4, C		106.3, C
H_3CO-6	3.75, s	60.0, CH_3	3.87, s	61.0, CH_3	3.87, s	60.9, CH_3
1′		121.1, C		123.3, C		123.2, C
2′	7.52, d (1.9)	115.9, CH	7.74, d (2.0)	117.3, CH	7.71, d (2.1)	117.3, CH
3′		144.9, C		145.9, C		145.9, C
4′		148.5, C		149.7, C		149.7, C
5′	6.81, d (8.5)	115.2, CH	6.89, d (8.5)	116.1, CH	6.87, d (8.5)	116.2, CH
6′	7.67, dd (8.6, 2.0)	122.0, CH	7.66, dd (8.5, 2.1)	123.3, CH	7.63, dd (8.5, 2.1)	123.2, CH
galactopyranose moiety						
1″	5.32, d (7.6)	101.7, CH	5.58, d (7.7)	101.3, CH	5.50, d (7.7).	101.3, CH
2″	3.17[c]	70.4, CH	3.99, t (8.1)	76.4, CH	3.97[c]	76.7, CH
3″	3.37[c]	73.0, CH	3.70[c]	75.4, CH	3.73[c]	75.3, CH
4″	3.64[c]	68.3, CH	3.83[c]	70.7, CH	3.85[c]	70.6, CH
5″	3.55[c]	74.4, CH	3.46[c]	77.1, CH	3.47, q (6.1)	77.0, CH
6″	3.67,[c] 3.37[c]	66.5, CH_2	3.47,[c] 3.60[c]	62.1, CH_2	3.62,[c] 3.50[c]	62.0, CH_2
apiopyranose moiety						
1‴			5.48, d (0.8)	110.4, CH	5.46, d (1.2)	110.7, CH
2‴			4.06, s	78.6, CH	4.06[c]	78.0, CH
3‴				80.1, C		80.8, C
4‴			3.68,[c] 4.10, d (9.8)	75.7, CH_2	3.70,[c] 4.04[c]	75.5, CH_2
5‴			3.63,[c] 4.10, d (9.8)	74.1, CH_2	3.62,[c] 3.75[c]	66.2, CH_2
arabinofuranose moiety						
1⁗	3.97, d (6.7)	102.6, CH	4.15, d (7.0)	105.2, CH		
2⁗	3.56[c]	71.0, CH	3.51[c]	72.4, CH		
3⁗	3.04[c]	72.5, CH	3.30[c]	74.1, CH		
4⁗	3.45[c]	67.4, CH	3.73[c]	69.6, CH		
5⁗	3.04,[c] 3.53[c]	65.0, CH_2	3.85,[c] 3.48[c]	66.9, CH_2		

[a]Recorded in DMSO-d_6. [b]Recorded in CD_3OD. [c]Signals overlapped, chemical shifts were derived from HSQC spectrum.

Extraction and Isolation. Air-dried aerial parts of *C. marschallii* were powdered and extracted using an ultrasonic bath at room temperature with an acetone−methanol−water (3:1:1) mixture (7 × 4 L). After concentration of the combined extracts at 45 °C, a sample was collected and freeze-dried for UHPLC analysis. The rest of the aqueous residue was fractionated by liquid−liquid extraction with chloroform (3 × 500 mL), diethyl ether (6 × 500 mL), ethyl acetate (6 × 500 mL), and finally *n*-butanol (7 × 400 mL). The combined layers were evaporated to dryness to yield 3.22 g of chloroform residue, 6.38 g of diethyl ether residue, 7.58 g of ethyl acetate residue, 25.17 g of *n*-butanol residue, and 16.27 g of aqueous residue. Afterward, to isolate the phenolics, the ethyl acetate and *n*-butanol residues were purified.

The EtOAc residue (7.50 g) was dissolved in a mixture of ethyl acetate and methanol, adsorbed onto silica gel (10.0 g), and subjected to chromatography on a silica gel column (150 mm × 50 mm) using an Interchim PuriFlash 430evo chromatograph. Elution was performed with a mixture of chloroform−methanol (from 100:0 to 0:100 in 12 steps; 0%, 5%, 7%, 10%, 15%, 27%, 35%, 45%, 55%, 75%, and 100% methanol, 300 mL per step). The flow rate was 15 mL/min. In total, 176 fractions were obtained and pooled into seven main fractions, A_1−A_7, based on TLC analysis (silica gel, ethyl acetate−formic acid−acetic acid−water, 100:11:11:26). Fraction A_4 was subjected to a Sephadex LH-20 column (79 cm × 3.2 cm) eluted with MeOH (isocratic flow). Altogether, 112 fractions were obtained, and they were grouped into 10 main fractions, B_1−B_{10}, based on TLC analysis (conditions as above). Fraction A_4B_6 was dissolved in DMSO for preparative HPLC (P1 method: Shimadzu LC-20AP instrument with a UV−vis detector, Kinetex SB-C_{18} (21.2 mm × 150 mm × 5 μm) column; flow rate 22 mL/min, mobile phase A: 0.1% HCOOH in H_2O, B: 0.1% HCOOH in CH_3CN; programmed gradient: 0−45 min 6−21% B, detection at 254 and 350 nm). This separation provided three compounds: patuletin 3-*O*-β-D-galactopyranoside (13.7 mg, t_R = 32.15−33.20 min), 6-methoxykaempferol 3-*O*-β-D-galactopyranoside (3.6 mg, t_R = 37.00−37.65 min), and 7 (23.6 mg, t_R = 38.20−40.05 min). Fraction A_5 was separated according to the same protocol as was used for fraction A_4, and the resulting 150 fractions were combined into main fractions C_1−C_6 based on TLC analysis. Fraction A_5C_3 was further separated by preparative HPLC (P1 method), which afforded pure naringenin 8-*C*-β-glucopyranoside (28.6 mg, t_R = 26.50−27.70 min).

The *n*-BuOH residue (25.0 g) was dissolved in water and separated on a Diaion HP-20 column (20 cm × 5 cm) using an Interchim PuriFlash 430evo device eluting with a gradient of H_2O−MeOH (from 100:0 to 0:100 in 10 steps; 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 50%, 75%, to 100%, 400 mL per step), and the flow rate was 15 mL/min.

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Table 3. IC_{50} Values Calculated for Isolated Compounds[a]

compound	ROS production IC_{50} ± SEM	IL-8 secretion IC_{50} ± SEM	TNF-α secretion IC_{50} ± SEM
1	26.9 ± 2.9	66.0 ± 12.1	
2	31.5 ± 7.4	25.7 ± 4.8	16.3 ± 5.5
3	5.7 ± 1.1	28.9 ± 12.1	9.3 ± 3.1
4	5.7 ± 0.7	25.5 ± 13.2	
5	5.4 ± 0.9	24.5 ± 5.3	9.7 ± 1.0
6	7.1 ± 1.4	7.2 ± 1.2	12.8 ± 4.5
7	38.5 ± 5.6	51.9 ± 4.7	16.3 ± 10.8
8	36.6 ± 3.3	46.7 ± 12.3	23.2 ± 13.1
9	6.0 ± 1.3		20.7 ± 5.6
6-methoxykaempferol 3-*O*-β-D-galactopyranoside	15.4 ± 3.2	18.1 ± 5.5	11.7 ± 3.9
naringenin 8-*C*-β-glucopyranoside	13.2 ± 3.5	11.4 ± 2.3	23.7 ± 6.6
patuletin 3-*O*-β-D-galactopyranoside	3.4 ± 0.6	8.0 ± 0.7	7.7 ± 1.5
quercetin (positive control)	4.4 ± 0.3	33.0 ± 11.6	20.4 ± 1.9

[a]Values given as μM.

In total, 28 fractions were obtained and combined into main fractions D_1−D_6, based on TLC analysis. Fraction D_3 was subjected to Toyopearl HW-40F column chromatography (55 cm × 2.2 cm) with an isocratic mobile phase (70% MeOH). In total, 48 fractions were collected, and they were combined into seven main fractions, E_1−E_7, based on TLC analysis. Fraction D_3E_3 was dissolved in DMSO and then subjected to preparative HPLC (P2 method: Shimadzu LC10vp chromatographic system (Kyoto, Japan) equipped with a UV−vis detector, Zorbax SB-C_{18} (21.2 × 150 mm × 5 μm) column, eluted with A: 0.1% HCOOH in H_2O, B: 0.1% HCOOH in CH_3CN at 5 mL/min using the following gradient: 0−60 min 3−26% B; 60−65 min 26−35% B, detection at 254 and 350 nm). This separation provided compounds **1** (42.2 mg, t_R = 24.30−30.70 min) and **2** (11.8 mg, t_R = 34.20−35.25 min). Fraction D_4 was separated using the same conditions as used for fraction D_3, with 60 fractions being obtained, which were pooled into five main fractions, F_1−F_5, based on TLC analysis. Four of the fractions were subjected to preparative HPLC: D_4F_2, D_4F_3, D_4F_4, and D_4F_5 were combined with other fractions featuring the same compounds. Dissolving fraction D_4F_2 in DMSO and subjecting it to the P2 method provided two pure compounds: **1** (40.0 mg, t_R = 29.85−31.00 min) and **2** (86.0 mg, t_R = 33.30−35.00 min). Preparative HPLC of fraction D_4F_3 dissolved in DMSO using the P1 method gave pure **3** (24.6 mg, t_R = 26.80−28.00 min), **4** (4.4 mg, t_R = 28.40−28.95 min), and **6** (17.6 mg, t_R = 29.90−31.20 min). Fraction D_4F_4 was combined with fraction D_3E_6, dissolved in DMSO, and separated by P2 to give pure patuletin 3-*O*-β-D-galactopyranoside (18.7 mg, t_R = 47.75−48.75 min). Combining D_4F_5 and D_3E_7, dissolving them in DMSO, and purifying the mixture by method P2 provided hyperoside (35.2 mg, t_R = 45.60−46.35 min). Fraction D_5 was separated on a Toyopearl HW-40F column (79 cm × 3.2 cm) eluted with 60% MeOH (isocratic flow). In total, 162 fractions were collected and pooled into seven main fractions, G_1−G_7, based on TLC analysis. Fraction D_5G_3 was dissolved in DMSO and separated using preparative HPLC according to method P1. As a result, four compounds were obtained: **2** (18.8 mg, t_R = 20.25−20.85 min), **4** (1.6 mg, t_R = 28.90−29.10 min), **5** (8.6 mg, t_R = 29.45−30.00 min), and **9** (2.1 mg, t_R = 43.60−43.90 min). Fraction D_5G_4 was purified by the same conditions used for D_5G_3 and gave five pure compounds: **3** (19.0 mg, t_R = 27.60−28.55 min), **5** (17.6 mg, t_R = 29.65−30.15 min), **6** (13.4 mg, t_R = 30.75−31.25 min), **8** (6.5 mg, t_R = 41.20−41.55 min), and **9** (7.0 mg, t_R = 43.65−43.90 min). A scheme of the isolation procedure is provided in Figure S2 (Supporting Information).

*Patuletin 3-O-β-D-galactopyranosyl-7-O-β-D-glucopyranoside (**1**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −62 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.41), 260 (4.23), 359 (4.14) nm; ^{1}H and ^{13}C NMR (DMSO-d_6), Table 1; HRESIMS *m*/*z* 655.1504 $[M - H]^-$ (calcd for $C_{28}H_{31}O_{18}^-$, 655.1510).

*Spinacetin 3-O-β-D-galactopyranosyl-7-O-β-D-glucopyranoside (**2**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −56 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.33), 257 (4.17), 354 (4.09) nm; ^{1}H and ^{13}C NMR (CD_3OD and DMSO-d_6), Table 1; HRESIMS *m*/*z* 669.1664 $[M - H]^-$ (calcd for $C_{29}H_{33}O_{18}^-$, 669.1667).

*Patuletin 3-O-(6″-O-β-D-glucopyranosyl)-β-D-galactopyranoside (**3**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −20 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.38), 259 (4.19), 359 (4.19) nm; ^{1}H and ^{13}C NMR (DMSO-d_6), Table 1; HRESIMS *m*/*z* 655.1510 $[M - H]^-$ (calcd for $C_{28}H_{31}O_{18}^-$, 655.1510).

*Patuletin 3-O-(6″-O-α-L-arabinopyranosyl)-β-D-galactopyranoside (**4**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −7 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 209 (3.41), 261 (3.09), 361 (3.01) nm; ^{1}H and ^{13}C NMR (DMSO-d_6), Table 2; HRESIMS *m*/*z* 625.1395 $[M - H]^-$ (calcd for $C_{27}H_{29}O_{17}^-$, 625.1405).

*Patuletin 3-O-(2″-O-(5‴-O-α-L-arabinopyranosyl)-β-D-apiofuranosyl)-β-D-galactopyranoside (**5**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −72 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.52), 260 (4.31), 355 (4.29) nm; ^{1}H and ^{13}C NMR (CD_3OD), Table 2; HRESIMS *m*/*z* 757.1826 $[M - H]^-$ (calcd for $C_{32}H_{37}O_{21}^-$, 757.1827).

*Patuletin 3-O-(2″-O-β-D-apiofuranosyl)-β-D-galactopyranoside (**6**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −128 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.34), 259 (4.04), 346 (3.98) nm; ^{1}H and ^{13}C NMR (CD_3OD), Table 2; HRESIMS *m*/*z* 625.1402 $[M - H]^-$ (calcd for $C_{27}H_{29}O_{17}$, 625.1405).

*Spinacetin 3-O-β-D-galactopyranoside (**7**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −58 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 214 (3.95), 257 (3.71), 271 (3.70), 332 (3.68) nm; ^{1}H and ^{13}C NMR (DMSO-d_6), Table 1; HRESIMS *m*/*z* 506.0985 $[M - H]^-$ (calcd for $C_{23}H_{23}O_{13}^-$, 507.1139).

*Patuletin 3-O-β-D-galactopyranosyl-7-O-(6‴-O-feruloyl)-β-D-glucopyranoside (**8**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −104 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.46), 260 (4.14), 273 (4.13), 330 (4.17) nm; ^{1}H and ^{13}C NMR (DMSO-d_6), Table 1; HRESIMS *m*/*z* 831.1975 $[M - H]^-$ (calcd for $C_{38}H_{39}O_{21}^-$, 831.1984).

*Spinacetin 3-O-β-D-galactopyranosyl-7-O-(6‴-O-feruloyl)-β-D-glucopyranoside (**9**):* pale yellow, amorphous solid; $[\alpha]^{20}_D$ −116 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.43), 255 (4.16), 274 (4.11), 332 (4.20) nm; ^{1}H and ^{13}C NMR (DMSO-d_6), Table 1; HRESIMS *m*/*z* 845.2141 $[M - H]^-$ (calcd for $C_{39}H_{41}O_{21}^-$, 845.2140).

Hydrolysis of the Glycosides and Determination of the Configurations of the Monosaccharides. Hydrolysis was performed as previously described[31] with some modification. Samples of the isolated glycosides were refluxed under acidic conditions only, for 2−12 h instead of 1 h. No additional purification (other than evaporation of water and HCl under reduced pressure) was performed. The configurations of the monosaccharides were determined as described in the previous report without changes.[31] Dry $CHCl_3$ extracts were dissolved in a CH_3CN−H_2O (20:80) mixture and filtered through a PTFE syringe filter. The derivatives obtained were analyzed by UHPLC-DAD-MS. Separations were performed on a Kinetex XB-C_{18} (150 mm × 3.0 mm × 2.6 μm) column eluted with A: 0.1% HCOOH in H_2O, B: 0.1% HCOOH in CH_3CN at a flow rate of 0.4 mL/min, isocratic flow 35% B, duration of the analysis 25 min. Samples were analyzed using extracted ion chromatograms that were obtained from the following ions: 537 amu for hexoses, 465 amu for pentoses, and 479 amu for rhamnose derivatives. Derivatives of the standards had retention times as follows: L-arabinose, t_R = 11.6 min; L-xylose, t_R = 12.0 min; D-arabinose, t_R = 12.2 min; D-apiose, t_R = 12.4 min; D-xylose, t_R = 12.5 min; D-galactose, t_R = 14.5 min; L-glucose, t_R = 16.4 min; D-glucose, t_R = 17.9 min: and L-rhamnose, t_R = 20.0 min. Comparing the ion masses and retention times of the derivatives of standards and derivatives of the hydrolyzed samples allowed for the identification of the saccharide moieties in the isolated glycosides. Based on the experiments described, D-glucose was identified in **1**, **2**, **3**, **8**, and **9**; D-galactose in **1**−**9**, patuletin 3-*O*-β-D-galactopyranoside, and 6-methoxykaempferol 3-*O*-β-D-galactopyranoside; L-arabinose in **4** and **5**; and D-apiose in **5** and **6**.

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Biological Activity Assays. Compounds **1**–**9**, patuletin 3-*O*-*β*-D-galactopyranoside, and 6-methoxykaempferol 3-*O*-*β*-D-galactopyranoside were dissolved in DMSO to prepare 10 mM stock solutions. Between experiments, the stock solutions were stored at −20 °C. Stock solutions were diluted in the appropriate solvents (HBSS or RPMI 1640) to obtain final concentrations of 5, 10, and 20 μM in the wells for the ROS-inhibition assays and 10, 20, and 50 μM for other assays. All isolated compounds used in the biological activity assays were >95% pure by HPLC. A positive control, quercetin, was purchased from Carl Roth (Karlsruhe, Germany) and was >99% pure by HPLC.

Isolation of Neutrophils and Determination of Their Viability. The buffy coat for isolation of human neutrophils was prepared as previously reported.[32] The viability of the neutrophils was determined by staining with propidium iodide (PI) using a previously reported protocol with few alterations.[32] Neutrophils (1×10^6/mL) were cultured in a 96-well plate in RPMI 1640 medium in the absence or presence of the investigated compounds at final concentrations of 10, 20, and 50 μM added 1 h before stimulation with LPS (100 ng/mL). Triton X-100 (0.1%) was used as a positive control.

ROS Production. The ROS production by f-MLP-stimulated neutrophils was determined using luminol-dependent chemiluminescence. The compounds were tested at concentrations of 5, 10, and 20 μM. Following isolation, the cells were suspended in HBSS. The cell suspension (2.0×10^5/mL) was incubated with 50 μL of the samples containing the tested compounds according to the previously reported protocol.[33]

IL-8 and TNF-*α* Production. Neutrophils (1.0×10^6/mL) for evaluation of IL-8 and TNF-α production were cultured in 96-well plates in RPMI 1640 medium with 10% fetal bovine serum, 10 mM HEPES, and 2 mM L-glutamine in the absence or presence of LPS (100 ng/mL) for 24 h at 37 °C with 5% CO_2. The cells were treated with the test compounds at final concentrations of 10, 20, and 50 μM in 1 mL of cell suspension (96-well plates, 1 mL per well) 1 h before stimulation. After 18 h, the solutions were centrifuged (2000 rpm; 10 min; 4 °C), and the supernatants were collected. The release of cytokines by stimulated neutrophils was evaluated by ELISA following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA, or R&D Systems, Minneapolis, MN, USA). Quercetin (10, 20, and 50 μM) was used as a positive control.

Statistical Analysis. The results are expressed as the mean ± SEM of the stated number of experiments. The statistical significances of the differences between the mean values were calculated using ANOVA analysis of variance with Tukey's post hoc test. Analyses were performed using STATISTICA software v. 13.1 PL (StatSoft, Kraków, Poland) and Excel 2016 (Microsoft Corporation, Redmond, WA, USA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00152.

Chromatogram of UHPLC-DAD-MS analysis, table with UV−vis and MS data, isolation scheme, scan of the voucher specimen, NMR spectra of all isolated compounds (PDF)

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Notes

The authors declare no competing financial interest.

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