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Flavoalkaloids and Flavonoids from *Astragalus monspessulanus*

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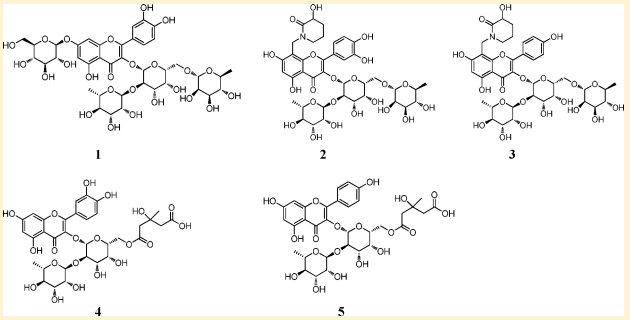
Supporting Information

ABSTRACT: A new flavonol tetraglycoside, quercetin-3-*O*-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl]-7-*O*-β-D-glucopyranoside (**1**), and two new flavonol alkaloids, *N*-(8-methylquercetin-3-*O*-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidin-2-one (**2**) and *N*-(8-methylkaempferol-3-*O*-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidin-2-one (**3**), were isolated from the aerial parts of *Astragalus monspessulanus* ssp. *monspessulanus*. Two rare flavonoids with an unusual 3-hydroxy-3-methylglutaric acid moiety, quercetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside (**4**) and kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside (**5**), were isolated from the aerial parts of *A. monspessulanus* ssp. *illyricus*. In addition, the eight known flavonoids alangiflavoside (**6**), alcesefoliside (**7**), mauritianin (**8**), quercetin-3-β-robinobioside (**9**), cosmosine (**10**), apigenin-4′-*O*-glucoside (**11**), trifolin (**12**), and rutin (**13**) were isolated from aerial parts of *A. monspessulanus* ssp. *monspessulanus*. Their structures were elucidated via NMR and HRESIMS data. In a model that tested *t*-BuOOH-induced oxidative stress on isolated rat hepatocytes, flavonoids **1**−**13** had statistically significant cytoprotective activity similar to that of silymarin, tested at 60 μg/mL. The most prominent effects were observed for flavonoids **1**, **4**, **7**, and **12**.



Astragalus is the largest genus in the plant family Fabaceae and comprises more than 2500 species widely distributed throughout the temperate regions of the world.[1] The genus is represented by 29 species in the flora of Bulgaria.[2] Several studies have suggested that *Astragalus* species possess interesting pharmacological properties including different protective, immunostimulant, antibacterial, antiviral, hepatoprotective, and other effects.[3−6] From a chemical point of view, the biologically active principles of *Astragalus* species represent saponins, flavonoids, and polysaccharides.[4,5] Flavonoids have been isolated from several species of the genus *Astragalus*.[4,5,7] In recent years, flavonol tri- and tetraglycosides acylated in the sugar chain were isolated from some *Astragalus* species.[8−13] Flavonol glycosides of kaempferol and methylkaempferol containing 3-hydroxy-3-methylglutaric acid in the sugar moiety were identified in *A. caprinus*[12] and *A.* gombiformis.[13] Those types of acylated compounds are rare in the plant kingdom and have otherwise only been found in some species of the genus *Rosa* (Rosaceae).[14] It was suggested that these rare flavonoids could be significant systematic markers for some sections of the genus.

Flavonoid alkaloids are an unusual group of structurally diverse secondary metabolites with varied pharmacological activities such as antineoplastic, immunomodulatory, and anti-inflammatory activities.[15] There are no data regarding the occurrence of flavoalkaloids in the genus *Astragalus*.

Astragalus monspessulanus L. (Fabaceae, Montpellier Milk Vetch) is a clump-forming perennial herb, 20−30 cm in height, with leaves composed of 21 to 41 oblong to ovate leaflets. The flowers are 2−3 cm long and purple and occur in an ovoid raceme. The plant has three subspecies: *A. monspessulanus* ssp. *monspessulanus* L., *A. monspessulanus* ssp. *illyricus* (Bernh.) Chater, and *A. monspessulanus* ssp. *teresianus* (Sennen & Elias) Amich. The plant is native to the Iberian Peninsula, France, Switzerland, the Apennine Peninsula, the Balkan Peninsula, Eastern Europe, and Northwest Africa.[1,2] Our previous studies showed that a purified saponin fraction, obtained from *A. monspessulanus* ssp. *monspessulanus*, possessed cytotoxicity in HepG2 cells.[16] During ongoing research it was found that the

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Table 1. NMR Spectroscopic Data (^{1}H 400 MHz and ^{13}C 100 MHz, Pyridine-d_5) for Compounds 1−3[a]

	1		2		3	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
2	158.1, C		157.2, C		157.1, C	
3	134.7, C		134.4, C		n.d.	
4	178.6, C		178.7, C		n.d.	
4a	107.0, C		105.1, C		105.3, C	
5	162.8, C		162.6, C		162.2, C	
6	100.2, CH	6.74, brs	99.4, CH	6.68, s	99.6, CH	6.69, s
7	163.6, C		165.1, C		165.4, C	
8	94.9, CH	6.87, brs	99.6, C		101.0, C	
8a	156.8, C		154.5,C		154.5, C	
1′	122.5, C		122.5, C		122.5, C	
2′	117.3, CH	8.32, brs	117.0, CH	8.48, d (2.0)	131.8, CH	8.71
3′	146.9, C		147.1, C		116.3, CH	7.30, d (8.7)
4′	150.7, C		150.4, C		161.5, C	
5′	116.4, CH	7.27, d (8.5)	116.5, CH	7.31, d (8.4)	116.3, CH	7.30, d (8.7)
6′	123.4, CH	8.36, d (8.5)	123.5, CH	8.56, dd (8.4, 2.0)	131.8, CH	8.71
1″			49.7, CH_2	4.53, d (14.1)	49.2, CH_2	4.40, d (13.8)
				4.39, d (14.1)		4.31
3″			174.9, C		176.6, C	
4″			67.6, CH	3.77, t (8.5)	66.1, CH	3.62
5″			29.7, CH_2	2.31, m	29.8, CH_2	2.24, m
				2.21, m		2.13, m
6″			23.6, CH_2	1.82, m	23.6, CH_2	1.76, m
				1.74, m		1.70, m
7″			53.8, CH_2	3.50	53.7, CH_2	3.29, td (8.5,2.8)
				2.81, q (8.1)		2.56, q (8.2)
Gal-1						
1	100.9, CH	6.42, d (7.8)	101.0, CH	6.40, d (7.7)	100.9, CH	6.44, d (7.8)
2	76.6, CH	4.95	76.6, CH	5.00, t (8.5)	76.4, CH	4.97
3	75.8, CH	4.33	75.9, CH	4.33	75.8, CH	4.32
4	70.1, CH	4.34	70.1, CH	4.29, s	70.1, CH	4.31, s
5	74.8, CH	4.08	75.1, CH	4.08	74.9, CH	4.08
6	66.1, CH_2	4.39	66.6, CH_2	4.28	66.5, CH_2	4.33
		3.90, dd (5.7, 9.8)		3.97, dd (6.0, 10.1)		3.92
Rha-2						
1	101.9, CH	5.17 brs	102.0, CH	5.13, brs	101.9, CH	5.14, brs
2	72.1, CH	4.33	72.1, CH	4.29	72.0, CH	4.32
3	72.6, CH	4.28	72.5, CH	4.27	72.5, CH	4.26
4	73.8, CH	4.12	73.8, CH	4.09	73.8, CH	4.09
5	69.6, CH	4.15	69.6, CH	4.13	69.5, CH	4.14
6	18.4, CH_3	1.44, d (6.6)	18.4	1.40, d (6.3)	18.4	1.41, d (6.0)
Rha-3						
1	102.5, CH	6.30, brs	102.5, CH	6.34, brs	102.4, CH	6.36, brs
2	72.8, CH	4.86, brs	72.8, CH	4.90	72.6, CH	4.88
3	72.8, CH	4.83	72.7, CH	4.89	72.8, CH	4.85
4	74.3, CH	4.28	74.3, CH	4.32	74.2, CH	4.28
5	69.9, CH	4.96	69.9, CH	5.06, m	69.9, CH	5.01
6	18.3, CH_3	1.59, d (6.4)	18.5, CH_3	1.65, d (6.4)	18.3, CH_3	1.59, d (6.2)
Glu-4						
1	101.7, CH	5.81, d (7.6)				
2	74.8, CH	4.33				
3	78.5, CH	4.38				
4	71.2, CH	4.32				
5	79.1, CH	4.13				
6	62.4, CH_2	4.54				
		4.40				

[a]The assignments were based on 1D ^{1}H and ^{13}C and 2D DQF-COSY, HSQC, and HMBC experiments. Multiplicity of obscured signals is not labeled. n.d. = not determined.

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n-butanol extract of *A. monspessulanus* ssp. *monspessulanus* exhibited hepatoprotective and antioxidant activities against in vitro and in vivo CCl_4-induced acute liver damage, comparable to that of silymarin.[17] In this study, we report the isolation of a new flavonol tetraglycoside (**1**), two new flavoalkaloids (**2**, **3**), and the eight known flavonoids alangiflavoside (**6**), alcesefoliside (**7**), mauritianin (**8**), quercetin-3-*β*-robinobioside (**9**), cosmosine (**10**), apigenin-4′-*O*-glucoside (**11**), trifolin (**12**), and rutin (**13**) from the same extract. In addition, two rare flavonol glycosides (**4**, **5**) of quercetin and kaempferol with an unusual 3-hydroxy-3-methylglutaric acid moiety were isolated from *A. monspessulanus* ssp. *illyricus*.

RESULTS AND DISCUSSION

Flavonoids from *A. monspessulanus* ssp. *monspessulanus*. Compound **1** was obtained as an orange, amorphous powder. The HRESIMS spectrum showed a molecular ion of the formic acid adduct $[M + HCOO]^-$ at m/z = 963.2650 (calcd 963.2612), establishing a molecular formula of $C_{39}H_{50}O_{25}$, when taken in conjunction with the ^{13}C NMR data. The NMR data of compound **1** (Table 1) suggested the presence of a quercetin tetraglycoside. Two of the sugar units were 6-deoxy sugars, while the other two were hexoses with C-5 hydroxymethylene groups. After complete resonance assignments and analyses of coupling constants, the intensities of cross-peaks in the COSY spectrum, and ^{13}C NMR chemical shift values, one hexose moiety was identified as a *β*-glucosyl unit, the second as a *β*-galactosyl moiety, while both 6-deoxy sugars were found to be *α*-rhamnosyl moieties. The ^{13}C NMR resonance values indicated that the glucosyl and both the rhamnosyl moieties were terminal sugar units. HMBC correlations were used to establish the structure of the side chains and their points of attachment to the aglycone. The anomeric proton (δ_H 5.81) of the glucose moiety showed a three-bond correlation to C-7 (δ_C 163.5) of the aglycone, while an HMBC correlation between the anomeric proton of the galactosyl moiety (δ_H 6.42) and the carbon at δ_C 134.7 indicated that the galactosyl unit was attached to C-3 of the aglycone. The anomeric protons of the rhamnose moieties (δ_H 5.17, δ_H 6.30) showed three-bond HMBC correlations to C-6 (δ_C 66.4) and C-2 (δ_C 76.6) of the galactosyl unit, respectively. Hence, compound **1** was identified as the new quercetin-3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranosyl]-7-*O*-*β*-D-glucopyranoside. It may be described as a 7-*O*-*β*-glucopyranoside of alcesefoliside (**8**), or it may be considered the quercetin counterpart of alangiflavoside (**6**).

Compound **2** was obtained as an orange, amorphous powder. The HRESIMS spectrum showed a deprotonated molecular ion $[M - H]^-$ at m/z = 882.2695 (calcd 882.2673), establishing a molecular formula of $C_{39}H_{49}NO_{22}$, when taken in conjunction with the ^{13}C NMR data. The proton and the HSQC NMR spectra of **2** (Table 1) revealed a flavone as aglycone with one *β*-galactosyl, two *α*-rhamnosyl units, and an additional moiety as side chains. The sugar units showed almost the same chemical shift values and the same HMBC correlations as those found in the spectra of compounds **1** and **6** for the sugar chains attached to C-3 for each of these compounds (i.e., compound **2** comprised one 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranosyl] moiety, too). Interestingly, the aglycone part of **2** was identified as quercetin with a C-bound aliphatic portion attached to C-8. The molecular mass of compound **2** indicated that the side chain contained a nitrogen atom, while the ^{13}C NMR chemical shift value (δ 49.7) and the doublet multiplicity of the proton resonances H-1″ suggested that this nitrogen atom was attached to C-1″. The remaining five carbon NMR resonances, which were derived from the 1D carbon spectrum, as well as from correlations in the 2D HMBC and multiplicity-edited HSQC spectra, comprised one carbonyl, one hydroxymethine, and three methylene groups. Cross-peaks in the DQF-COSY spectrum between the methine proton H-4″ (δ 3.77) and the methylene protons H-5″ (δ 2.31, 2.21), methylene protons H-5″ and H-6″ (δ 1.82, 1.74), and methylene protons H-6″ and H-7″ (δ 3.50, 2.81) were observed. In addition, HMBC correlations were observed between H-4″ and C-3 (δ 174.9) and between H-5″ and C-3 (δ 174.9). The three-bond HMBC correlations between the H-1″ protons (δ 4.53, 4.39) and C-3 (δ 174.9) and C-7″ (δ 53.8), respectively, suggested that these two C atoms were also attached to the nitrogen atom, leading to the molecular structure as depicted. This structure was supported by a proton spectrum of **2** recorded in DMSO-d_6: only one hydroxy resonance, namely, that of the 5-OH, was observed at low field, suggesting the formation of a lactam functionality. Hence, compound **2** was assigned as *N*-(8-methylquercetin-3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranosyl])-3-hydroxypiperidin-2-one. Formally, compound **2** can be considered a new oxidation product of a flavonoid with an *N*-methyl-3-hydroxypiperidin-2-one. The occurrence of flavonoid alkaloids is quite rare, with only a few dozen compounds known, but these have been well documented.[15] However, this is the first time that a natural product with an *N*-methyl-3-hydroxypiperidin-2-one moiety has been described.

Compound **3** was obtained as a pale yellow, amorphous powder. The HRESIMS spectrum showed a deprotonated molecular ion $[M - H]^-$ at m/z = 866.2747 (calcd 866.2724), establishing a molecular formula of $C_{39}H_{49}NO_{21}$, when taken in conjunction with the ^{13}C NMR data. The only major difference in the ^1H and HSQC NMR spectra of compound **3** as compared to compound **2** was the added number of aromatic resonances indicating the presence of kaempferol as an aglycone in compound **3** (Table 1). The observed sugar resonances were similar to the resonances of compound **2**, and HMBC correlations were identical with the corresponding data of compound **2**. Hence, compound **3** was assigned as *N*-(8-methylkaempferol-3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranosyl])-3-hydroxypiperidin-2-one.

The two flavone alkaloids **2** and **3** showed remarkably high chemical shift value differences for some of the carbon resonances of the piperidin-2-one system: for the C-1″ methylene groups the $\Delta\delta_C$ was found to be 0.5 ppm, for the C-3″ carbonyls 1.7 ppm, and for the hydroxymethine groups C-4″ 1.5 ppm, respectively. The most likely explanation for these chemical shift value differences is a differing spatial orientation of the piperidin-2-one with respect to the hydroxy/dihydroxy B-rings of their respective aglycones due to the formation of a hydrogen bond between the piperidin-2-one carbonyl and the 3′-OH group in compound **2**. Comparisons between the chemical shift differences of protons H-2′ and H-6′ in compounds **2** and **3** with their parent compounds alcesefolioside (**7**) and mauritianin (**8**) (data not shown) support the assumption of an interaction of the piperidin-2-one and phenyl rings: comparing compounds **2** and alcesefolioside (**7**) shows a $\Delta\delta_H$ of 0.2 ppm for H-2′ and H-6′, comparing compounds **3**

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and mauritianin (**8**) shows a $\Delta\delta_H$ of 0.15 ppm, while other positions like H-6 show only small values for $\Delta\delta_H$.

All other flavonoids were identified by comparing the experimental and reported ^{1}H and ^{13}C NMR data with alangiflavoside (**6**),[18] alcesefoliside (**7**),[19] mauritianin (**8**),[20] quercetin-3-*β*-robinobioside (**9**),[21] cosmosine (**10**),[22] apigenin-4′-*O*-glucoside (**11**),[23] trifolin (**12**),[24] and rutin (**13**).[25]

Flavonoids from *A. monspessulanus* ssp. *illyricus*. On the basis of their MS values, $[M - H]^-$ at m/z = 753.1899 (calcd for $C_{33}H_{37}O_{20}$ 753.1884) (**4**), $[M - H]^-$ at m/z = 737.1948 (calcd for $C_{33}H_{37}O_{19}$ 737.1935) (**5**), and NMR spectroscopic properties (Table 2), compounds **4** and **5** were identified as the rare flavonoid glycosides quercetin-3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-*β*-D-galactopyranoside and kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-*β*-D-galactopyranoside, respectively.[14]

Table 2. NMR Spectroscopic Data (^{1}H 400 MHz and ^{13}C 100 MHz, Pyridine-d_5) for Compounds 4 and 5[a]

	4		5	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
2	157.5, C		157.2, C	
3	134.3, C		134.2, C	
4	178.7, C		n.d.	
4a	105.5, C		105.5, C	
5	163.0, C		162.9, C	
6	99.6, CH	6.69, d (1.8)	99.7, CH	6.71, d (2.0)
7	165.6, C		165.6, C	
8	94.4, CH	6.64, d (1.8)	94.5, CH	6.77, d (2.0)
8a	157.6, C		157.5, C	
1′	122.8, C		122.2, C	
2′	117.2, CH	8.27, d (2.1)	131.8, CH	8.54, d (8.6)
3′	146.9, C		116.2, CH	7.23
4′	150.5, C		161.4, C	
5′	116.4, CH	7.30, d (8.4)	116.2, CH	7.23
6′	123.4, CH	8.39, dd (2.1, 8.4)	131.8, CH	8.54, d (8.6)
Gal-1				
1	100.6, CH	6.47, d (7.8)	100.5, CH	6.47 d (7.7)
2	76.6, CH	4.93	76.5, CH	4.94
3	75.7, CH	4.28	75.6, CH	4.29
4	70.3, CH	4.27	70.1, CH	4.27
5	74.1, CH	4.10, t (6.4)	74.1, CH	4.11, t (6.3)
6	63.9, CH_2	4.62	63.9, CH_2	4.63
		4.62		4.63
Rha-2				
1	102.5, CH	6.27, brs	102.5, CH	6.30, brs
2	72.8, CH	4.84	72.7, CH	4.85
3	72.9, CH	4.80	72.9, CH	4.80
4	74.3, CH	4.26	74.2, CH	4.28
5	70.1, CH	4.96	70.0, CH	4.94
6	18.4, CH_3	1.58, d (6.3)	18.3, CH_3	1.55, d (6.3)
HMG				
	171.3, C		171.1	
	46.3, CH_2	3.02	46.3, CH_2	3.01
		2.95		2.96
	70.0, C		69.8, C	
	46.3, CH_2	2.98	46.3, CH_2	2.98
		2.91		2.91
	174.6, C		174.5, C	
	28.1, CH_3	1.63, s	28.3, CH_3	1.61, s

[a]The assignments were based on 1D ^{1}H and ^{13}C and 2D DQF-COSY, HSQC, and HMBC experiments. Multiplicity of obscured signals is not labeled. n.d. = not determined.

While the presence of flavone glycosides was expected from published data on Bulgarian *Astralagus* species, the isolation of flavoalkaloids and flavonol esters of 3-hydroxy-3-methylglutaric acid from *A. monspessulanus* was surprising. The latter were until now considered to be chemosystematically characteristic compounds of *Rosa* species;[14] however, they seem to be present also in other genera.[12,13] On the other hand, there are significant differences in the flavonoid composition between the two subspecies of *A. monspessulanus*. Flavonol alkaloids (**2** and **3**) and flavonoid tetraglycosides (**1** and **6**) were isolated only from ssp. *monspessulanus*, while flavonols acylated with 3-hydroxy-3-methylglutaric acid (**4** and **5**) were found in ssp. *illyricus*. Comparative HPLC analysis showed that mauritianin, alcesefoliside, trifolin, and rutin are found in both subspecies. These chemical characteristics, as well as the morphological differences between the subspecies, can be used to support the identification of plant material collected from the wild and also could solve systematic problems.

The isolated flavonoids **1**−**13** were studied for possible hepatoprotective and antioxidant effects in a test system to evaluate *t*-BuOOH-induced oxidative stress at three different concentrations: 0.6, 6, and 60 *μ*g/mL. The effects of the compounds were compared with those of silymarin as positive control. The highest concentration (60 *μ*g/mL) revealed the most prominent cytoprotective effect on cell viability in *t*-BuOOH toxicity. Incubation of hepatocytes with 75 *μ*M *t*-BuOOH resulted in a statistically significant reduction of cell viability by 74% ($p < 0.001$), as compared to the control. In combination with *t*-BuOOH, the examined flavonoids at a concentration of 60 *μ*g/mL caused a statistically significant preservation of the cell viability. Compound **1** preserved cell viability by 186% ($p < 0.001$); **2**, by 159% ($p < 0.01$); **3**, by 177% ($p < 0.001$); **4**, by 182% ($p < 0.01$); **5**, by 155% ($p < 0.01$); **6**, by 164% ($p < 0.01$); **7**, by 195% ($p < 0.001$); **8**, by 168% ($p < 0.05$); **9**, by 159% ($p < 0.01$); **10**, by 177% ($p < 0.001$); **11**, by 159% ($p < 0.01$); **12**, by 191% ($p < 0.01$); and **13**, by 168% ($p < 0.01$), as compared to the *t*-BuOOH group. Silymarin (60 *μ*g/mL) preserved cell viability by 182% ($p < 0.01$) as compared to the *t*-BuOOH group (Figure 1).

The results showed that in the model of *t*-BuOOH-induced oxidative stress flavonoids **1**−**13** had statistically significant hepatoprotective and antioxidant activities, similar to those of silymarin (Figure 1). The most prominent were the effects observed for flavonoids **1**, **4**, **7**, and **12**. Flavonoids **7** and **12** had stronger protective effects than that of silymarin on cell viability. The cytoprotective effects of these compounds on *t*-BuOOH-induced oxidative stress were possibly related to their activity as scavengers of reactive oxygen species (ROS), and these effects were comparable to the effects of silymarin, a well-known ROS scavenger.[26]

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were collected in absolute MeOH on a Beckman Coulter DN 800 spectrophotometer. NMR spectra were recorded on a Varian Unity Inova spectrometer operating at a proton frequency of 400 MHz. All compounds were dissolved in 0.72 mL of pyridine-d_5 with 0.1% TMS as an internal standard; in addition, a proton spectrum of compound **2** was recorded in DMSO-d_6.

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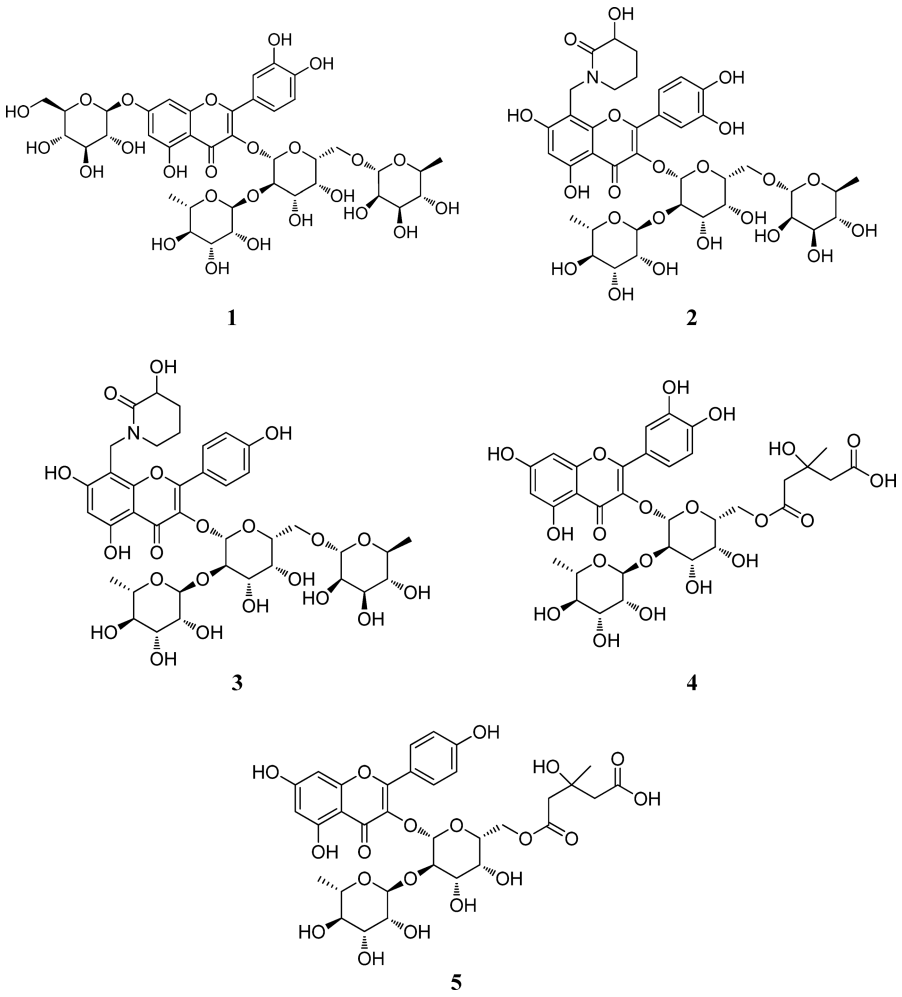
Chart 1



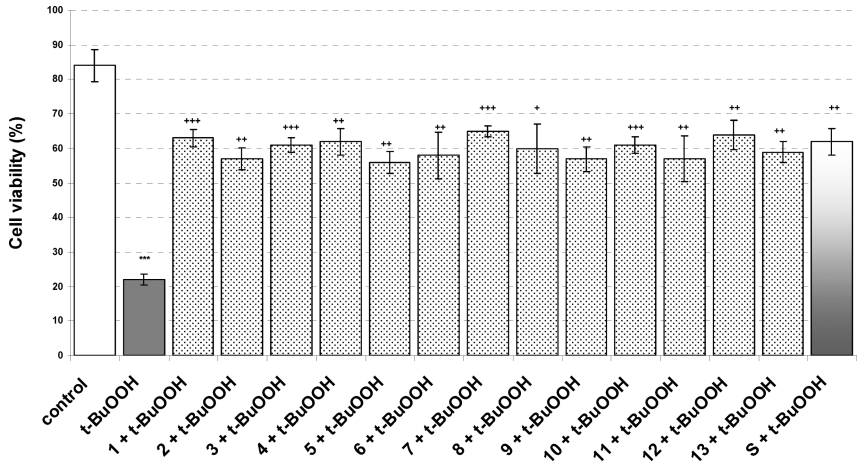


Figure 1. Effect of flavonoids **1**–**13** (60 μg/mL) as compared to silymarin (60 μg/mL) on the cell viability of isolated rat hepatocytes in *tert*-butyl hydroperoxide (75 μM *t*-BuOOH)-induced oxidative stress; $^{***}p < 0.001$ vs control (nontreated hepatocytes); $^{++}p < 0.01$; and $^{+++}p < 0.001$ vs *t*-BuOOH.

The 1D ^{1}H and ^{13}C and 2D DQF-COSY, HSQC, and HMBC experiments were performed at 25 °C with standard pulse programs from the Varian user library. Accurate mass determinations were performed using an LC/FTMS system consisting of an Exactive Orbitrap mass spectrometer, equipped with a nonheated ESI source (ThermoFisher Scientific, Inc., Bremen, Germany) and operated in ultrahigh-resolution mode (100.000) coupled to a U-HPLC system (Accela, ThermoFisher Scientific, Inc.). Operating conditions for the ESI source used in the negative ionization mode were 4.6 kV spray voltage, 250 °C capillary temperature, sheath gas flow rate 50 units, and auxiliary gas flow 5 units (units refer to arbitrary values set by the Exactive software). Nitrogen was used for sample nebulization. U-HPLC separations were performed on a Hypersil Gold C_{18} (ThermoFisher Scientific, USA), 1.9 μm, 2.1 × 50 mm i.d., HPLC

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column, operated at 30 °C. Each 10 min chromatographic run was carried out at a flow rate of 0.3 mL/min with a binary mobile phase consisting of MeOH + 0.1% HCOOH (A) and 10 mM NH_4COOH + 0.1% HCOOH (B) using a step gradient profile of 50% A for 0.5 min, increasing up to 100% A in 5 min, held isocratic at 100% for 0.5 min, then brought back down to 50% A over 0.1 min. After re-equilibration at 50% A for 3.9 min, the next sample was injected.

TLC was carried out on precoated silica gel plates (Kieselgel G, F_{254}, 60, Merck, Darmstadt, Germany) with the solvent systems EtOAc−HCOOH−H_2O (10:1:4), EtOAc−HCOOH−AcOH−H_2O (32:3:2:6), and EtOAc−EMK−HCOOH−H_2O (5:3:1:1). Spots were visualized under UV light (365 nm) by spraying with NTS/PEG reagent. Column chromatography (CC) was performed using Diaion HP-20 (Supelco, USA) and Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden). For low-pressure liquid chromatography (LPLC), silica gel (MN Kieselgel 60, 0.04−0.063 mm, Macherey-Nagel, Düren, Germany) and octadecylsilyl (ODS) gel DAVISIL (Grace Davison Discovery Sciences, Hesperia, CA, USA) were used. Semipreparative HPLC was performed on a Waters (Milford, MA, USA) high-pressure binary gradient system consisting of a pump model 1525EF, manual injector 7725i, UV detector model 2489, and the software Breeze 2, using a Luna prepacked semipreparative ODS column (100 Å, 250 × 10 mm, 5 μm, Phenomenex, Torrance, CA, USA) and eluting with H_2O−*o*-H_3PO_4 0.05%−MeCN at a constant flow rate of 4.5 mL/min. For HPLC analysis, a Luna prepacked ODS column (100 Å, 250 × 4.6 mm, 5 μm, Phenomenex) eluted with H_2O−*o*-H_3PO_4 0.05%−MeCN at a constant flow rate of 1 mL/min was used. All chromatograms were monitored at 254 and 330 nm.

GC-MS analysis of (2*R*)-2-butyl glycosides was performed using an Agilent 7890A GC system interfaced to an Agilent 5975C MSD operating at 70 eV, ion source temperature 230 °C, interface temperature 280 °C. A split injection (1 μL injection volume, split ratio, 20:1) at 270 °C injector temperature was utilized. A fused silica capillary column, 5% phenyl/95% methyl polysiloxane (HP-5MS 30 m × 250 μm × 0.25 μm, Agilent J & W, USA), was used. The temperature program was as follows: 100 °C, then 270 °C at 3 °C/min. The carrier gas was helium 5.6 at a flow rate of 1.4 mL/min. Data acquisition was performed with Agilent GC/MSD ChemStation version E.02.02 for the mass scan range 40−600 u.

The chemicals used in the pharmacology experiments were pentobarbital sodium (Sanofi, France); NaCl, KCl, $NaHCO_3$, $CaCl_2 \cdot 2H_2O$, and D-glucose (Merck, Germany); KH_2PO_4 (Scharlau Chemie SA, Spain); $MgSO_4 \cdot 7H_2O$ (Fluka AG, Germany); HEPES, collagenase from *Clostridium histolyticum* type IV, albumin, bovine serum fraction V minimum 98%, EGTA, *tert*-butyl hydroperoxide (Sigma-Aldrich, Germany).

Plant Material. The aerial parts of *A. monspessulanus* ssp. *monspessulanus* were collected in May 2010 from Rodopi Mountain close to the town of Dzhebel, Bulgaria, at coordinates 41°32′56.00″ N, 25°21′47.43″ E, whereas the aerial parts of *A. monspessulanus* ssp. *illyricus* were collected in May 2013 from a rocky area in the western parts of Stara Planina Mountain close to the town of Vratsa, Bulgaria, at coordinates 43°8′5.31″ N, 23°29′9.23″ E. The plants were identified by Dr. D. Pavlova from the Department of Botany, Faculty of Biology, Sofia University, where voucher specimens have been deposited (*A. monspessulanus* ssp. *monspessulanus*, N SO 107533, and *A. monspessulanus* ssp. *illyricus*, N SO 107532).

Extraction and Isolation. The air-dried powdered plant material from *A. monspessulanus* ssp. *monspessulanus* (280 g) was exhaustively extracted with 80% MeOH under reflux (23 × 750 mL). The extracts were filtered and concentrated under reduced pressure. The hydrophilic residue was dissolved in H_2O and extracted with CH_2Cl_2 to remove the lipophilic constituents. The defatted water residue was successively extracted with EtOAc and *n*-BuOH. The *n*-BuOH extract was evaporated to dryness to give a solid residue (53.6 g). Part of the extract (50 g) was subjected to CC over Diaion HP-20 (4.7 × 45 cm), eluting with H_2O−MeOH (100:0 → 0:100, v/v), to give nine main fractions (I−IX). Fraction II was chromatographed over Sephadex LH-20, eluting with MeOH, and six subfractions were collected (C1−C6). Subfraction C3 was subjected to LPLC over an ODS column (2.4 × 30 cm), eluting with MeOH−H_2O (32:68, v/v), followed by isocratic semipreparative HPLC, eluting with a mobile phase of MeCN−H_2O (10:90, v/v), to give compounds **1** (12 mg) and **6** (11 mg). Subfraction C2 afforded compounds **2** (13 mg) and **3** (5.5 mg) using the same separation procedure as that used for fraction C3. Fraction III was chromatographed over Sephadex LH-20 (4.7 × 50 cm), eluting with MeOH, to give six subfractions (A1−A6). Subfraction A4 was purified by repeated LPLC over ODS C_{18} (2.4 × 30 cm), eluting with MeOH−H_2O (40:60, v/v), and was further subjected to isocratic semipreparative HPLC, eluting with the mobile phase MeCN−H_2O (14:86, v/v), to give compound **7** (45 mg). Fraction IV was purified by CC over Sephadex LH-20, eluting with MeOH, and four subfractions were collected (B1−B4). Further separation of subfraction B2 was performed by repeated LPLC over an ODS column (2.4 × 30 cm), eluting with MeOH−H_2O (35:65, v/v), followed by isocratic semipreparative HPLC, eluting with the mobile phase MeCN−H_2O (14:86, v/v), which afforded compound **8** (48 mg). In a similar manner to that described for subfraction B2, subfraction B3 was fractionated to yield compounds **9** (17.4 mg) and **13** (5.1 mg).

The EtOAc extract (5.56 g) was chromatographed over a silica gel column, eluting with a gradient of CH_2Cl_2−MeOH-H_2O (90:10:0 → 80:20:3, v/v/v), to obtain 13 fractions (Et1−Et13). Fraction Et4 was purified by repeated LPLC over an ODS column, eluting with a gradient of MeOH−H_2O (50:50 → 0:100, v/v), and five subfractions were collected ($Et4_1$−$Et4_5$). Subfraction $Et4_4$ was further subjected to isocratic semipreparative HPLC, eluting with a mobile phase of MeCN−H_2O (19:81, v/v), to obtain compounds **10** (4.5 mg), **11** (6.1 mg), and **12** (4.8 mg).

The air-dried plant material of *A. monspessulanus* ssp. *illyricus* (95 g) was subjected to extraction with CH_2Cl_2 to remove the lipophilic constituents. The defatted plant material was exhaustively extracted with a gradient of MeOH−H_2O (100:0 → 80:20, v/v) at room temperature. The extract was filtered, and the solvent was removed under reduced pressure, yielding 19.6 g of solid residue. The residue was suspended in H_2O (100 mL) and fractionated by CC with Diaion HP-20 (4.7 × 45 cm) to give six main fractions (A−F). Fraction C was subjected to repeated LPLC over an ODS column (2.4 × 30 cm), eluting with MeOH−H_2O (30:70, v/v), and four subfractions were collected (C1−C4). Subfraction C2 was purified by CC over Sephadex LH-20 (2.5 × 50 cm) followed by isocratic semipreparative HPLC, eluting with the mobile phase MeCN−H_2O (17:83, v/v), to afford compounds **4** (29 mg) and **5** (6 mg).

Quercetin-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl]-7-O-β-D-glucopyranoside (**1**): orange, amorphous powder; UV (MeOH) λ_{max} (log ε) 250 (4.04), 263 (sh) (3.87), 351 (3.74) nm; ^{1}H NMR (pyridine-d_5, 400 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 100 MHz), see Table 1; HREIMS *m*/*z* 963.2650 (calcd. for $C_{40}H_{51}O_{27}$, 963.2612 [M + HCOO]$^-$).

N-(8-Methylquercetin-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidin-2-one (**2**): orange, amorphous powder; UV (MeOH) λ_{max} (log ε) 251 (4.13), 263 (sh) (3.97), 293 (sh) 3.60), 355 (3.96) nm; ^{1}H NMR (pyridine-d_5, 400 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 100 MHz), see Table 1; HREIMS *m*/*z* 882.2695 (calcd for $C_{39}H_{48}NO_{22}$, 882.2673 [M − H]$^-$).

N-(8-Methylkaempferol-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidin-2-one (**3**): pale yellow, amorphous powder; UV (MeOH) λ_{max} (log ε) 259 (4.05), 293 (sh) (3.82), 345 (3.99) nm; ^{1}H NMR (pyridine-d_5, 400 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 100 MHz), see Table 1; HREIMS *m*/*z* 866.2747 (calcd. for $C_{39}H_{48}NO_{21}$, 866.2724 [M − H]$^-$).

Determination of Absolute Configuration of Sugars. The analysis was carried out by GC-MS according to the method by Reznicek et al. including acidic hydrolysis and preparation of (2*R*)-2-butyl glycosides.[27] Compounds **1**−**5** (2 mg) were hydrolyzed with concentrated AcOH (3.5 mL), concentrated HCl (1 mL), and H_2O (5.5 mL) at 100 °C for 2 h. The mixture was extracted with EtOAc,

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and the remaining aqueous phase evaporated to dryness. (2*R*)-2-BuOH (0.45 mL) and concentrated HCl (0.1 mL) were added to the residue and heated to 100 °C for 15 h. The samples were evaporated to dryness in a stream of N_2, and TMS derivatives were prepared by adding 100 μL of Sigma-Sil-A (Sigma-Aldrich, Germany). Standard compounds L-rhamnose, D-glucose, and D-galactose (Sigma-Aldrich, Germany) were treated by the same procedure. The (2*R*)-2-butyl glycosides were separated by GC-MS analysis, resulting in peaks at t_R = 20.74, 22.35 (L-Rha), 28.80, 29.71 (D-Gal), and 29.87, 31.94 (D-Glc) min, respectively. Compound **1** revealed L-Rha, D-Gal, and D-Glc; in compounds **2**–**5** L-Rha and D-Gal were present.

Hepatoprotective and Antioxidant Bioassay. The male Wistar rats (body weight 200–250 g) were housed in Plexiglas cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20 ± 2 °C and humidity 72 ± 4%) with free access to water and standard pelleted rat food 53-3, produced according ISO 9001:2008. Animals were purchased from the National Breeding Center, Sofia, Bulgaria. At least 7 days of acclimatization were allowed before the commencement of the study. The health of animals was monitored regularly by a veterinary physician. The vivarium (certificate of registration of farm No. 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (No. A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) (Council of Europe, 1991) and were strictly followed throughout the experiment. For the isolation of hepatocytes, rats were anesthetized with sodium pentobarbital (0.2 mL/100 g). An optimized in situ liver perfusion using fewer reagents and shorter time of cell isolation was performed. The method provided a higher amount of live and metabolically active hepatocytes.[28] After portal catheterization, the liver was perfused with HEPES buffer (pH = 7.85) + 0.6 mM EDTA (pH = 7.85), followed by HEPES buffer (pH = 7.85) and finally HEPES buffer containing collagenase type IV (50 mg/200 mL) and 7 mM $CaCl_2$ (pH = 7.85). The liver was excised and minced into small pieces, and the hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) + 1% bovine serum albumin. Cells were counted under the microscope, and the viability was assessed by Trypan blue exclusion (0.05%).[29] Initial viability averaged 89%. Cells were diluted with KRB to make a suspension of about 3×10^6 hepatocytes/mL. Incubations were carried out in flasks containing 3 mL of the cell suspension (i.e., 9×10^6 hepatocytes) and were performed in a 5% CO_2 + 95% O_2 atmosphere.

Statistical analysis was performed using the statistical program MEDCALC. Results are expressed as mean ± SEM for six experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. A level of $p < 0.05$ was considered significant. Three parallel samples were used. The IC_{50} was calculated using GraphPad Prism Statistical Software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00502.

^1H, ^{13}C, DQF-COSY, HSQC, HMBC, and HRESIMS spectra of compounds **1**–**3** (PDF)

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Notes

The authors declare no competing financial interest.

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