Glycosides from *Cephalaria* Species

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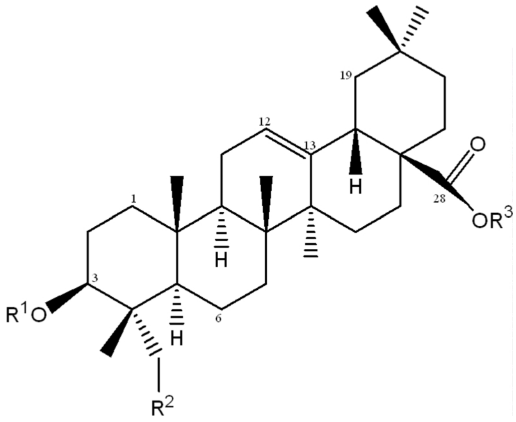
Three novel triterpene glycosides (**1** – **3**), namely lycicoside I, II and cilicicoside I, were isolated from two different *Cephalaria* (Dipsacaceae) species along with one known oleanane- and one iridoit-type of glycoside. The structures of these compounds were established as 3-*O*-[β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl(1→4)-β-D-xylopyranosyl]-28-*O*-[β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl]-oleanolic acid (**1**), 3-*O*-[β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid (**2**) from *Cephalaria lycica* Matthew and 3-*O*-{β-D-glucopyranosyl(1→4)-β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-α-L-rhamnopyranosyl}-28-*O*-[β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl]-hederagenin (**3**) from *Cephalaria cilicica* Boiss. & Kotschy, on the basis of spectroscopic methods (1D and 2D NMR techniques, mass spectrometry) and chemical evidence. In addition, three new prosapogenins, **1B** – **3B**, were obtained from the basic hydrolysis of **1** – **3**. The antimicrobial activity of **1** – **3** was tested against some Gram-positive and Gram-negative bacteria strains.

Key words: Dipsacaceae, *Cephalaria*, Oleanane and Hederagenin Glycosides, Lycicoside I and II, Cilicicoside I

Introduction

The *Cephalaria* genus comprises about 93 species which are widespread in Europe, East Asia, East Mediterranea, North and Central Africa. There are 39 species which are spread out extensively in Turkey, and 23 of them are endemic [1]. Throughout the ages, many plants have been the basis of traditional medicines [2]. *Cephalaria* species have been used as folk medicine for many years due to their alleviative, anti-infective, hypothermic and relaxant activities [3, 4]. The chemical constituents previously reported to be found in these plants were terpenoids, iridoids, flavonoids, alkaloids, and lignans [5 – 18]. Some of these natural products from *Cephalaria* genus have antimicrobial, antifungal, antioxidant, cytotoxic, and insecticidal activities. Because of these properties, they are used for medical, agricultural and veterinary purposes [19 – 26]. Therefore, we decided to make further investigations on *Cephalaria* species using our knowledge from earlier studies which had resulted in the isolation of several natural products. In this paper, the isolation and structure determination of three new glycosides and prosapogenins along with a known triterpene (Scoposide A) [27] and an iridoid-type glycoside [28] are



	R1	R2	R3
1	Glc(1→3)Rha(1→4)Xyl(1→4)Xyl-	H-	Glc(1→6)Glc-
2	Xyl(1→3)Rha(1→4)Xyl-	H-	Glc-
3	Glc(1→4)Xyl(1→3)Rha(1→2)Rha- ↓3 Glc	HO-	Glc(1→6)Glc-
1B	Glc(1→3)Rha(1→4)Xyl(1→4)Xyl-	H-	H-
2B	Xyl(1→3)Rha(1→4)Xyl-	H-	H-
3B	Glc(1→4)Xyl(1→3)Rha(1→2)Rha- ↓3 Glc	HO-	H-

Fig. 1. Structures of compounds **1** – **3** and **1B** – **3B**.

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Table 1. ^{1}H NMR spectral data (δ) of compounds **1**–**3** ([D$_6$]DMSO)[a].

Aglycon	**1**	**2**	**3**	**3-O-Sugars**	**1**	**2**	**3**
3	2.97 m	2.99 m	3.44 m	H-1′	4.24 d 7.2	4.24 d 7.2	5.06 br s
5	0.67 s	0.67 s	1.16 s	H-2′	2.99 m	3.54 m	3.47 m
9	1.47 m	1.45 m	1.48 m	H-3′	3.51 m	3.18 m	3.58 m
12	5.11 br s	5.13 br s	5.15 br s	H-4′	3.53 m	3.55 m	3.56 m
23	0.92 s	0.92 s	3.31, 3.34 s	H-5′	3.35, 3.64 m	3.34, 3.61 m	4.24 m
24	0.73 s	0.73 s	0.55 s	H-6′			1.03 d 6.4
25	0.84 s	0.84 s	0.86 s	H-1″	4.25 d 7.2	5.12 br s	5.09 br s
26	0.65 s	0.66 s	0.66 s	H-2″	3.52 m	3.78 m	3.48 m
27	1.05 s	1.05 s	1.07 s	H-3″	3.01 m	3.60 m	3.63 m
29	0.84 s	0.84 s	0.85 s	H-4″	2.93 m	3.41 m	3.38 m
30	0.84 s	0.84 s	0.84 s	H-5″	3.35, 3.64 m	3.56 m	3.57, m
				H-6″		1.06 d 6.0	1.08 d 5.6
				H-1‴	5.14 br s	4.28 d 7.6	4.36 d 7.6
				H-2‴	3.79 m	3.06 m	3.58 m
				H-3‴	3.60 m	3.18 m	3.21 m
				H-4‴	3.40 m	3.11 m	3.43 m
				H-5‴	3.56, m	3.64, 3.68 m	3.34, 3.68 m
				H-6‴	1.06 d 6.4		
				H-1⁗	4.30 d 7.6		4.29 d 8.0
				H-2⁗	3.11 m		nd
				H-3⁗	3.01 m		3.47 m
				H-4⁗	3.79 m		3.94 m
				H-5⁗	3.14 m		3.19 m
				H-6⁗	3.40, 3.62 m		3.40, 3.44 m
				H-1′′′′′			4.31 d 8.0
				H-2′′′′′			2.89 m
				H-3′′′′′			3.21 m
				H-4′′′′′			3.13 m
				H-5′′′′′			3.17 m
				H-6′′′′′			3.61 m
				28-C-Sugars			
				H-1′′′′′′	5.20 d 8.0	5.20 d 8.0	5.21 d 8.0
				H-2′′′′′′	3.10 m	3.08 m	3.11 m
				H-3′′′′′′	3.36 m	3.07 m	2.99 m
				H-4′′′′′′	3.17 m	3.11 m	3.67 m
				H-5′′′′′′	nd	3.10 m	3.09 m
				H-6′′′′′′	3.88 m	3.40, 3.59 m	3.89, 3.78 m
				H-1′′′′′′′	4.17 d 8.0		4.18 d 8.0
				H-2′′′′′′′	3.23 m		2.91 m
				H-3′′′′′′′	3.17 m		3.02 m
				H-4′′′′′′′	3.01 m		3.02 m
				H-5′′′′′′′	3.51 m		3.09 m
				H-6′′′′′′′	3.63, 3.41 m		3.63 m

[a] Assignments were based on COSY, HMQC and HMBC experiments; nd, not determined.

described. The isolated compounds **1**–**3** (Fig. 1) were also evaluated for antimicrobial activity against some Gram-positive and Gram-negative bacteria.

Results and Discussion

The *n*-BuOH fractions of the methanolic extract of *C. lycica* and *C. cilicica* were subjected to chromatographic studies, and further purifications afforded three new triterpene glycosides (**1**–**3**), and three new prosapogenins (**1B**–**3B**) along with two known glycosides [27, 28]. Their structures were established by IR spectroscopy, ESI-MS, HR-ESI-MS and mainly by 2D NMR techniques.

Compound **1** was obtained as a colorless amorphous powder. The HR-ESI-MS of **1** established its molecular composition as $C_{64}H_{104}O_{30}Na$. This compound showed in the ESIMS (positive-ion mode) a molecular ion peak at *m/z* = 1375.6 $[M+Na]^+$. The IR spectrum revealed absorption bands at 3381.7, 1738.9, 1640.6, and 1077.1 cm^{-1} which corresponded to -OH, -C=O, -C=C and -C-O-C groups, respectively. Complete as-

Table 2. ^{13}C NMR spectral data (δ) of compounds **1** – **3** ([D$_6$]DMSO)[a].

Aglycon	**1**	**2**	**3**	**3-O-Sugars**	**1**	**2**	**3**
1	nd	39.1	39.0	C-1$'$	102.3	104.7	100.7
2	26.3	26.5	25.6	C-2$'$	73.4	73.7	78.6
3	88.5	88.5	88.1	C-3$'$	77.0	77.3	82.3
4	39.1	39.4	42.9	C-4$'$	74.5	74.3	71.1
5	55.9	55.9	46.9	C-5$'$	65.3	65.3	68.1
6	19.2	18.4	18.0	C-6$'$			18.6
7	32.9	32.9	32.6	C-1$''$	104.8	100.3	100.5
8	39.4	39.7	42.0	C-2$''$	73.6	70.6	74.9
9	47.8	47.8	47.8	C-3$''$	77.4	81.7	82.3
10	37.0	37.0	36.7	C-4$''$	74.1	71.6	71.3
11	23.2	23.6	24.7	C-5$''$	65.3	68.7	68.5
12	122.3	122.3	122.5	C-6$''$		18.4	18.5
13	144.2	144.1	144.2	C-1$'''$	100.4	106.4	104.8
14	41.9	42.0	41.4	C-2$'''$	70.6	74.4	73.9
15	28.0	28.0	28.0	C-3$'''$	81.7	77.3	76.4
16	23.6	23.2	23.5	C-4$'''$	71.6	70.2	78.6
17	46.6	46.6	46.7	C-5$'''$	68.7	66.4	65.6
18	41.4	36.5	nd	C-6$'''$	18.4		
19	46.3	46.3	46.3	C-1$''''$	106.0		103.9
20	30.9	30.9	30.9	C-2$''''$	74.3		72.9
21	33.9	33.9	34.0	C-3$''''$	77.4		73.7
22	32.3	31.5	31.9	C-4$''''$	70.8		69.9
23	28.1	28.0	63.2	C-5$''''$	77.6		77.3
24	17.0	17.0	13.7	C-6$''''$	61.8		61.7
25	15.9	15.9	16.3	C-1$'''''$			102.8
26	17.4	17.3	17.4	C-2$'''''$			74.3
27	26.2	26.2	26.2	C-3$'''''$			76.3
28	175.9	175.9	175.9	C-4$'''''$			69.9
29	33.5	33.4	33.4	C-5$'''''$			77.2
30	24.1	24.0	24.1	C-6$'''''$			61.3
				28-C-Sugars			
				C-1$''''''$	94.7	94.8	94.7
				C-2$''''''$	72.9	73.1	73.1
				C-3$''''''$	77.2	77.0	77.1
				C-4$''''''$	69.9	70.1	71.5
				C-5$''''''$	77.6	78.4	75.8
				C-6$''''''$	68.6	61.3	68.5
				C-1$'''''''$	103.7		103.7
				C-2$'''''''$	75.0		74.2
				C-3$'''''''$	77.2		77.5
				C-4$'''''''$	70.8		70.7
				C-5$'''''''$	77.0		77.4
				C-6$'''''''$	61.7		61.7

[a] Assignments were based on COSY, HMQC and HMBC experiments; nd, not determined.

signment of all proton and carbon resonances in the ^{1}H and ^{13}C NMR spectra of **1** was achieved using COSY, HMQC and HMBC data (Tables 1 and 2). In the ^{1}H NMR spectrum, seven methyl singlets at δ = 1.05, 0.92, 0.84, 0.84, 0.84, 0.73, and 0.65, an olefinic proton at δ = 5.11, and six anomeric protons at δ = 5.20, 5.14, 4.30, 4.25, 4.24, and 4.17 were observed. Full assignment of the ^{1}H and ^{13}C NMR spectra of **1**, using 1D and 2D data sets, equivalent with those acquired for **2**, confirmed that both saponins were oleanane-type triterpenoids. However, **1** had two more glucose units than **2**. The remaining quaternary carbons (C-4, C-8, C-10, C-14, C-17 and C-20) were successfully analyzed by comparative HMBC and HMQC data. For the carbohydrate units, the anomeric carbons were observed at 102.3 (C-1$'$), 104.8 (C-1$''$), 100.4 (C-1$'''$), 106.0 (C-1$''''$), 94.7 (C-1$''''''$), and 103.7 (C-1$'''''''$) ppm, and the anomeric protons at δ = 4.24 (1H, *d*, J = 7.2 Hz), 4.25 (1H, *d*, J = 7.2 Hz), 5.14 (1H, *br s*), 4.30 (1H, *d*, J = 7.6 Hz), 5.20 (1H, *d*, J = 8.0 Hz), and 4.17 (1H, *d*, J = 8.0 Hz), indicating the presence of xylose, xylose, rhamnose, glycose and two more glycose moieties, respectively. The absolute configurations of the monosaccharide constituents of **1** were obtained by acid hydrolysis. These configurations were confirmed to be α for rhamnose and β for xylose and glucose. Using the correlations in the HMBC spectra, sugar-sugar and sugar-aglycone linkages were specified. In the HMBC spectrum of **1**, the specific correlation between H-1′ (δ = 4.24) and C-3 (δ = 88.5) confirmed that the xylose unit was linked to the aglycon at the C-3 position. As it was seen for **2**, a downfield shift of C-3 supported the glycosylation of 3-OH. Another correlation between H-1′′′′′ (δ = 5.20) and C-28 (δ = 175.9) pointed to a linkage between glucose and the carbonyl carbon (C-28) of the aglycon. These findings were also corroborated by the NMR spectra of the alkaline hydrolysis product. In this spectrum, the peaks that belonged to two glucose units had disappeared. Therefore, we understood that there was a sugar unit which was linked to the C-28 position, and realized that compound **1** was a bisdesmosidic triterpene glycoside. The downfield-shifted resonance of C-6′′′′′ (δ = 68.6), belonging to glucose, indicated further substitution at this position, as confirmed by HMBC correlations between H-1′′′′′′ (δ = 4.17) and C-6′′′′′ (δ = 68.6). Thus, a β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl moiety was found to have an ester linkage at C-28. The remaining sugar correlations between H-1′′ (δ = 4.24) and C-4′ (δ = 74.5), H-1′′′ (δ = 5.14) and C-4′′ (δ = 74.1), and H-1′′′′ (δ = 4.30) and C-3′′′ (δ = 81.7) ensured the designation of the linkages between the remaining sugars at the C-3 position. COSY and HMBC data also confirmed the internal structure of the carbohydrate units. On the basis of the above results, the structure of **1** was elucidated as 3-*O*-[β-D-glucopyranosyl (1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl(1→4)-β-D-xylopyranosyl]-28-*O*-[β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl]-oleanolic acid (lycicoside I).

Compound **2** was obtained as a colorless amorphous powder. The molecular formula of this compound was deduced as $C_{52}H_{84}O_{20}$ from the ESI-MS, HR-ESI-MS and 2D NMR data. The positive ESI-MS of **2** revealed a sodiated molecular ion peak at *m/z* = 1051.7 $[M+Na]^+$. The negative MS/MS of the molecular ion showed the main fragment peaks at *m/z* = 865.5 $[M–Glc]^-$, 733.5 $[M–Glc–Xyl]^-$, 587.4 $[M–Glc–Xyl–Rhm]^-$, and 455.2 $[M–Glc–Xyl–Rhm–Xyl]^-$due to the sequential loss of carbohydrate units. The IR spectrum revealed absorption bands at 3571.4, 3397.8, 1657.1, 1603.0, and 1045.3 cm^{-1} which correspond to -OH, -C-H, -C=O, -C=C and -C-O-C groups, respectively. In the 1D NMR data of **2** (Table 1), seven methyl singlets at δ = 1.05, 0.92, 0.84, 0.84, 0.84, 0.73, and 0.66, one olefinic proton at δ = 5.13 (1H, *br s*) and a specific signal of H-3 at δ = 2.99 (1H, *m*) belonged to the aglycon. In addition, in the ^{13}C NMR spectrum, while the peak of the quaternary carbon C-28 was seen at 175.9 ppm, two characteristic olefinic carbon atoms were observed at 122.3 for C-12 and at 144.1 ppm for C-13. These values confirmed the aglycon skeleton (Table 2). The remaining quaternary carbons (C-4, C-8, C-10, C-14, C-17, and C-20) were successfully analyzed by comparative HMBC and HMQC data. For carbohydrate units, the anomeric carbons were observed at 104.7 (C-1′), 100.3 (C-1′′), 106.4 (C-1′′′), and 94.8 (C-1′′′′) ppm, and the anomeric protons were detected at δ = 4.24 (1H, *d*, *J*= 7.2 Hz), 5.12 (1H, *br s*), 4.28 (1H, *d*, *J*= 7.6 Hz), and 5.20 (1H, *d*, *J* = 8.0 Hz). These anomeric protons indicated the presence of xylose, rhamnose, xylose, and glycose moieties, respectively. Taking into account their coupling constants, the acidic hydrolysis process and GC-MS results, the absolute configuration of the sugars was deduced to be α for rhamnose and β for glycose and the two xylose moieties. The sugar-sugar and sugar-aglycone linkages were obtained from the HMBC spectrum. According to the data, the correlation between H-1′′′′ (δ = 5.20) and C-28 (δ = 175.9) indicated that glucose was connected to the aglycon at the carbonyl carbon (C-28). This finding was confirmed by an alkaline hydrolysis. Another correlation between H-1′ (δ = 4.24) and C-3 (δ = 88.5) confirmed that the xylose unit was linked to the aglycon at the C-3 position. A downfield shift of C-3 supported the glycosylation of 3-OH. This finding was associated with the ^1H NMR spectrum of the alkaline hydrolysis product which showed that the peaks belonging to glucose had disappeared. Thus, it was understood that the sugar which was linked to position C-28 was glycose, and the compound was established as a bisdesmosidic triterpene glycoside. Other sugar correlations between H-1′′ (δ = 5.12) and C-4′ (δ = 74.3), and between H-1′′′ (δ = 4.28) and C-3′′ (δ = 81.7), ensured the designation of the linkage between the remaining sugars at the C-3 position. COSY and HMBC spectra also confirmed the internal structure of the carbohydrate units.

These observations were used to assign the structure of **2** as a new bisdesmosidic triterpene glycoside, namely 3-*O*-[β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid (lycicoside II).

Compound **3** was isolated as a colorless amorphous powder. The molecular formula, $C_{71}H_{116}O_{36}$, was determined using 1D, 2D NMR, ESI-MS, HR-ESI-MS and IR experiments. In the positive-mode ESI-MS spectrum, the sodiated molecular ion peak appeared at *m/z* = 1568.2 $[M+Na]^+$. The negative-ion HR-ESI-MS of this compound revealed a peak at *m/z* = 1543.7179 $[M]^-$ (calcd. 1543.7174 for $C_{71}H_{115}O_{36}$). The IR spectrum gave specific signals for -OH, -C=O, -C=C and -C-O-C groups at 3418.2, 1621.4, 1596.7, and 1059.02 cm^{-1}. In the ^1H NMR spectrum specific peaks of six methyl groups were observed at 1.07, 0.86, 0.85, 0.84, 0.66, and 0.55 ppm as singlets. These peaks indicated that the type of this aglycon is hederagenin. Also, the signals of the olefinic proton (H-12) at δ = 5.15 (1H, *br s*) and of H-3 at δ = 3.44 (1H, *m*) clearly proved this type of aglycon. In addition, the anomeric protons of the sugars were observed at δ = 5.06 (1H, *br s*) for rhamnose, at δ = 5.09 (1H, *br s*) for another rhamnose, at δ = 4.36 (1H, *d*, *J* = 7.6 Hz) for xylose, at δ = 4.29 (1H, *d*, *J* = 8.0 Hz) for the first glucose, at δ = 4.31 (1H, *d*, *J* = 8.0 Hz) for the second glucose, at δ = 5.21 (1H, *d*, *J* = 8.0 Hz) for the third glucose, and at δ = 4.18 (1H, *d*, *J* = 8.0 Hz) for the fourth glucose. In the ^{13}C NMR spectrum, peaks of a quaternary carbonyl carbon (C-28) at 175.9 ppm and of two olefinic carbons at 122.5 (C-12) and at 144.2 (C-13) ppm were observed as specific signals for the aglycon. The remaining quaternary carbons (C-4, C-8, C-10, C-14, C-17, and C-20) were found using HMBC and HMQC data. The anomeric carbons were observed at 100.7 (C-1′), 100.5 (C-1′′), 104.8 (C-1′′′), 103.9 (C-1′′′′), 102.8 (C-1′′′′′), 94.7 (C-1′′′′′′), and 103.7 (C-1′′′′′′′) ppm for rhamnose, rhamnose, xylose and four glucose units, respectively. The HMBC spectrum was used for verifying the structure of the aglycon. The correlation of the $-CH_2-$ group (C-23) with the $-CH_3$ group (C-24) indicated that the aglycon was hederagenin. The correlation between C-28 and H-1′′′′′′ clarified that glucose (δ = 94.7) was connected to the aglycon at the carbonyl carbon (C-28). Another specific correlation between C-3 and H-1′ implied that rhamnose was linked to the aglycon at the C-3 position. These linkages were affirmed by basic hydrolysis. The ^1H NMR spectrum of the basic hydrolysis product showed that the peaks which belonged to two glucose units had disappeared, proving that two glucose units were connected to each other and linked to the C-28 position, and that the compound was a bisdesmosidic triterpene glycoside. The downfield-shifted resonances of C-6′′′′′′ (δ = 68.5) belonging to glucose indicated further substitution at this position. This linkage was also confirmed by an HMBC correlation between H-1′′′′′′′ (δ = 4.18) and C-6′′′′′′ (δ = 68.5). Thus, it became evident that a β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl moiety had an ester linkage at C-28. The remaining sugar correlations between H-2′ (δ = 3.47) and C-1′′ (δ = 100.5), H-1′′′ (δ = 4.36) and C-3′′ (δ = 82.3), H-4′′′ (δ = 3.43) and C-1′′′′ (δ = 103.9), and H-3′ (δ = 3.58) and C-1′′′′′ (δ = 102.8), ensured the designation of the linkages between the remaining sugars at the C-3 position. COSY and HMBC data were also consistent with the internal structure of the carbohydrate units.

Table 3. Antimicrobial activities of compounds **1**–**3** (MIC, $\mu g\ mL^{-1}$).

P. aeruginosa	32	64	16	2
S. typhimurium	32	16	16	1
K. pneumonie	32	64	32	4
S. aureus	64	64	16	1
S. epidermidis	64	32	16	1
B. cereus	32	16	32	4
E. faecalis	32	16	32	16

According to all these results, the exact structure of this novel bisdesmosidic glycoside was determined to be 3-*O*-{β-D-glucopyranosyl(1→4)-β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-α-L-rhamnopyranosyl}-28-*O*-[β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl]-hederagenin (cilicicoside I).

The alkaline hydrolysis of compounds **1**–**3** afforded three new prosapogenins, namely 3-*O*-[β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl(1→4)-β-D-xylopyranosyl]-oleanolic acid (**1B**), 3-*O*-[β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl]-oleanolic acid (**2B**), and 3-*O*-{β-D-glucopyranosyl(1→4)-β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-α-L-rhamnopyranosyl}-hederagenin (**3B**). The structures of these prosapogenins were identified by NMR and MS data.

Compounds **1**–**3** were tested for their antimicrobial activities by the MIC method against some Gram-positive and several Gram-negative bacteria. From the antimicrobial test results (Table 3) it appears that compound **3** exhibits the highest antimicrobial activity against both Gram-positive and Gram-negative bacteria. The remaining compounds **1** and **2** revealed only moderate activities, lower in comparison to that of **3** (Table 3).

Conclusion

Three new glycosides (**1** – **3**) were obtained from the *n*-butanolic extract of *C. lycica* and *C. cilicica*, and three new prosapogenins of these compounds (**1B** – **3B**) were identified by chemical methods. We hope that these findings will be helpful and directive for us and/or other scientists in the future research of *Cephalaria* species.

Experimental Section

General procedures

Optical rotations were measured on a Rudolph Research Analytical Autopol I automatic polarimeter. IR spectroscopy was performed on an ATI Mattson Genesis Series FT-IR spectrophotometer. Mass analyses were performed by a Bruker HCT Ultra ESI-MS ion trap instrument in positive mode. HR-ESI-MS measurements were run on a Bruker LC micro-Q-TOF instrument, and NMR experiments were performed on a Varian AS 400 MHz spectrometer. Standard pulse sequences and parameters were used to obtain 1D and 2D NMR spectra. Chemical shifts are referenced to the residual signal of [D_6]DMSO converted to TMS. GC-MS analysis was performed by an HP 6890-5973 instrument with an HP-5MS column. Medium-pressure liquid chromatography (MPLC) was carried out using a Buchi system (Büchi C-605 pumps, UV detector) with Büchi glass columns (15/460 and 49/230). Reverse-phase silica gel Lichroprep RP-18 (Merck 9303) was used for vacuum liquid chromatography (VLC), while silica gel 60 (Merck 7734) was used for open CC and MPLC experiments. Pre-coated silica gel $60F_{254}$ (Merck 5554) and reverse-phase silica gel RP-18 F_{254S} (Merck 5560) aluminum plates were used for TLC. After developing with a solvent system $CHCl_3$-MeOH-H_2O (from 90 : 10 : 1 to 61 : 32 : 7 with increasing polarity), the plates were visualized under UV light (254 and 366 nm) and sprayed with 20 % H_2SO_4 solution in water followed by heating at 120 °C for 3 min.

Plant materials

C. lycica Matthew was collected from Gömbe, Antalya, rocky places of the Girdev plateau and *C. cilicica* Boiss. & Kotschy from Finike-Elmalı vicinity, Antalya, Yalnız village, wayside, in July, 2007. They were identified by Prof. Dr. H. Sümbül and Assoc. Prof. R. S. Göktürk. Voucher specimens (R. S. Göktürk 6073 and R. S. Göktürk 6076) have been deposited at the Herbarium Research and Application Center of Akdeniz University, Antalya, Turkey.

Extraction and isolation

Aerial parts of *C. lycica* were dried at r. t. in the shade for a week. The dried and powdered plant (1.27 kg) was extracted with MeOH (3 × 4500 mL) at r. t. Each extraction was treated for one night. The MeOH extracts were put together, and solvents were evaporated by rotary evaporatoration at ∼ 40 °C under reduced pressure to yield a crude extract (252.9 g), 100.0 g of which was extracted with *n*-BuOH-H_2O (1 : 1, 3 × 200 mL). After the separation of the *n*-BuOH and H_2O phases had been completed, the H_2O phase was treated with *n*-BuOH (5 × 100 mL), and the *n*-BuOH phase was treated with H_2O (3 × 100 mL). Then all *n*-BuOH phases were combined and extracted with hexane (5 × 25 mL) in order to remove apolar components. This re-purified *n*-BuOH extract (33.8 g) was chromatographed by VLC using RP silica gel Lichroprep RP-18 (230 g) with a gradient of MeOH-H_2O (from 100 % H_2O to 100 % MeOH) to give 12 main fractions. First, fraction 10 (4.85 g) was subjected to an open silica gel CC and eluted with a gradient of $CHCl_3$-MeOH-H_2O (70 : 30 : 3 – 61 : 32 : 7) to give 20 subfractions. Subfraction 20 (485 mg) was subjected to open silica gel CC and eluted with a gradient of $CHCl_3$-MeOH-H_2O (80 : 20 : 2 – 61 : 32 : 7) to give 7 fractions. Fraction 6 (370 mg) was re-chromatographed on a silica gel column using $CHCl_3$-MeOH-H_2O (90 : 10 : 1 – 61 : 32 : 7) to furnish compound **1** (270 mg). A second isolation process was applied on another subfraction of the first column (7th) (101.2 mg) with open silica gel CC eluting with a gradient of $CHCl_3$-MeOH-H_2O (70 : 30 : 3 – 61 : 32 : 7) to afford compound **2** (81.7 mg). The final isolation process for *C. lycica* was carried out with the combined subfractions 11 and 12 (230 mg). These were subjected to silica gel CC eluting with a gradient system of $CHCl_3$-MeOH-H_2O (80 : 20 : 2 – 61 : 32 : 7) to yield compound **4** (105.6 mg) [27].

The same procedures that were described for *C. lycica* were performed for obtaining the *n*-BuOH extract (34.5 g) of *C. cilicica*. Fifteen main fractions were obtained from the *n*-BuOH extract by VLC using RP silica gel Lichroprep RP-18 (230 g). First, combined fractions 10 (4.25 g) and 11 (1.87 g), which showed great similarity, were subjected to MPLC over silica gel 60 using a suitable column and program (max. pressure: 20 bar, flow rate: 30 mL/min, CH_2Cl_2-MeOH solvent system, from 0 % MeOH to 100 % MeOH, 11 segments, 15 min per segment), and it afforded 8 fractions. Fractions 7 (2.06 g) and 8 (259 mg) were combined and applied to an open silica gel CC with $CHCl_3$-MeOH-H_2O (61 : 32 : 7) to afford compound **3** (84.0 mg). The second and the last isolation process for *C. cilicica* was carried out with the main VLC fractions 3 and 4. These were combined (1.2 g) and subjected to an open silica gel CC. Elution was carried out with $CHCl_3$-MeOH-H_2O (90 : 10 : 1) yielding compound **5** (170 mg) [28].

*Compound **1***

Colorless amorphous powder; 270 mg; $[\alpha]_D^{25} = -1.37$ (c = 1.46, MeOH). – IR (KBr): ν = 3382 (OH), 1739 (C=O), 1641 (C=C), 1077 (C-O-C) cm^{-1}. – ^{1}H NMR (400 MHz, [D$_6$]DMSO, TMS) and ^{13}C NMR (100 MHz, [D$_6$]DMSO, TMS) data: see Tables 1 and 2, respectively. – MS (ESI, 20 eV): *m/z* = 1375.6 $[M+Na]^+$. – HRMS ((-)-ESI): *m/z* = 1375.6523 (calcd. 1375.6505 for $C_{64}H_{104}O_{30}Na$, $[M+Na]^+$) (Fig. 1).

*Compound **2***

Colorless amorphous powder; 81.7 mg; $[\alpha]_D^{25} = -2.02$ (c = 1.14, MeOH). – IR (KBr): ν = 3571 (OH), 3397 (CH), 1657 (C=O), 1603 (C=C), 1045 (C-O-C) cm^{-1}. – ^{1}H NMR (400 MHz, [D$_6$]DMSO, TMS) and ^{13}C NMR (100 MHz, [D$_6$]DMSO, TMS) data: see Tables 1 and 2, respectively. – MS (ESI, 20 eV): *m/z* = 1051.7 $[M+Na]^+$. – HRMS ((-)-ESI): *m/z* = 1027.5441 (calcd. 1027.5483 for $C_{52}H_{83}O_{20}$, $[M]^-$) (Fig. 1).

*Compound **3***

Colorless amorphous powder; 84 mg; $[\alpha]_D^{25} = -3.42$ (c = 0.65, MeOH). – IR (KBr): ν = 3418 (OH), 1621 (C=O), 1596 (C=C), 1059 (C-O-C) cm^{-1}. – ^{1}H NMR (400 MHz, [D$_6$]DMSO, TMS) and ^{13}C NMR (100 MHz, [D$_6$]DMSO, TMS) data: see Tables 1 and 2, respectively. – MS (ESI, 70 eV): *m/z* = 1568.2 $[M+Na]^+$. – HRMS ((-)-ESI): *m/z* = 1543.7179 (calcd. 1543.7174 for $C_{71}H_{115}O_{36}$, $[M]^-$) (Fig. 1).

Alkaline hydrolysis

Solutions of compounds **1** – **3** (15 mg of each) in 5 % aqueous KOH solution were refluxed for 1 h at 80 °C. Then the solution was neutralized with 5 % aqueous HCl solution [27]. The evaporated residues were extracted with *n*-BuOH-H_2O (1 : 1, 6 mL). The organic layers of the alkaline hydrolysis of the pure compounds afforded three new prosapogenins (**1B** – **3B**).

*3-O-[β-D-Glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl(1→4)-β-D-xylopyranosyl]-oleanolic acid (**1B**)*

Colorless amorphous powder; 7.9 mg; $[\alpha]_D^{25} = +3.77$ (c = 0.53, MeOH). – ^{1}H NMR (400 MHz, [D$_6$]DMSO, TMS): aglycon: δ_H = 3.0 (m, 1H, 3-H), 0.68 (s, 1H, 5-H), 1.43 (m, 1H, 9-H), 5.15 (br s, 1H, 12-H), 0.96 (s, 3H, 23-H), 0.72 (s, 3H, 24-H), 0.87 (s, 3H, 25-H), 0.68 (s, 3H, 26-H), 1.08 (s, 3H, 27-H), 0.89 (s, 3H, 29-H), 0.85 (s, 3H, 30-H); sugars: δ_H = 4.26 (d, J = 7.2 Hz, 1H, H-1′), 4.27 (d, J = 7.2 Hz, 1H, H-1′′), 5.16 (br s, 1H, H-1′′′), 4.31 (d, J = 7.6 Hz, 1H, H-1′′′′). – MS (ESI, 20 eV): *m/z* = 1027.5 $[M]^+$. – HRMS ((+)-ESI): *m/z* = 1027.5487 (calcd. 1027.5483 for $C_{52}H_{83}O_{20}$, $[M]^+$).

*3-O-[β-D-Xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-xylopyranosyl]-oleanolic acid (**2B**)*

Colorless amorphous powder; 9.0 mg; $[\alpha]_D^{25} = +2.67$ (c = 0.75, MeOH). – ^{1}H NMR (400 MHz, [D$_6$]DMSO, TMS): aglycon: δ_H = 3.01 (m, 1H, 3-H), 0.69 (s, 1H, 5-H), 1.47 (m, 1H, 9-H), 5.18 (br s, 1H, 12-H), 0.94 (s, 3H, 23-H), 0.75 (s, 3H, 24-H), 0.83 (s, 3H, 25-H), 0.70 (s, 3H, 26-H), 1.06 (s, 3H, 27-H), 0.84 (s, 3H, 29-H), 0.85 (s, 3H, 30-H); sugars: δ_H = 4.25 (d, J= 7.2 Hz, 1H, H-1′), 5.09 (br s, 1H, H-1′′), 4.27 (d, J = 7.6 Hz, 1H, H-1′′′). – MS (ESI, 20 eV): *m/z* = 865.5 $[M]^-$. – HRMS ((-)-ESI): *m/z* = 865.4910 (calcd. 865.4955 for $C_{46}H_{73}O_{15}$, $[M]^-$).

*3-O-{β-D-Glucopyranosyl(1→4)-β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-α-L-rhamnopyranosyl}-hederagenin (**3B**)*

Colorless amorphous powder; 5.9 mg; $[\alpha]_D^{25} = +5.12$ (c = 0.39, MeOH). – ^{1}H NMR (400 MHz, [D$_6$]DMSO, TMS): aglycon: δ_H = 3.46 (m, 1H, 3-H), 1.17 (s, 1H, 5-H), 1.45 (m, 1H, 9-H), 5.21 (br s, 1H, 12-H), 3.33, 3.36 (2 × m, 2H, 23a-H; 23b-H), 0.55 (s, 3H, 24-H), 0.85 (s, 3H, 25-H), 0.69 (s, 3H, 26-H), 1.06 (s, 3H, 27-H), 0.83 (s, 3H, 29-H), 0.82 (s, 3H, 30-H); sugars: δ_H = 5.06 (br s, 1H, H-1′), 5.09 (br s, 1H, H-1′′), 4.36 (d, J = 7.6 Hz, 1H, H-1′′′), 4.29 (d, J = 8.0 Hz, 1H, H-1′′′′), 4.31 (d, J = 8.0 Hz, 1H, H-1′′′′′). – MS (ESI, 20 eV): *m/z* = 1219.6 $[M]^-$. – HRMS ((-)-ESI): *m/z* = 1219.6125 (calcd. 1219.6117 for $C_{59}H_{95}O_{26}$, $[M]^-$).

Acid hydrolysis and GC-MS analysis

The carbohydrate units of the compounds were determined using micro-hydrolysis and GC-MS methods [14, 29]. The pure compounds **1** – **3** were applied to a TLC layer (silica gel 60 F_{254}) and treated with concentrated HCl vapor in a closed vessel saturated at 60 °C for 40 min. When the hydrolysis treatment was finished, the reference sugars (glucose, galactose, arabinose, xylose, rhamnose, mannose, and fucose) were applied to the TLC layer, and the TLC was developed by $CHCl_3$-MeOH-H_2O-gAcOH (16 : 9 : 2 : 2). By spraying with (2 %) α-naphthol-H_2SO_4 solution, and heating the plate at 120 °C, the carbohydrate units were identified. For GC-MS analysis each compound (5 mg) was hydrolyzed with 1 N HCl (2 mL) in 80 % MeOH-benzene (1 : 1) (2.5 mL) solution under reflux for 6 h at 95 °C. After extraction with $CHCl_3$ (3 × 5 mL), the aqueous layer was evaporated to dryness and then analyzed by TLC. The residue of sugars was dissolved in anhydrous pyridine (1 mL), and then 1 mL of HMDS-TMCS (hexamethyldisilazane-trimethylchlorosilane

1 : 1) was added for silylation. The mixture was stirred at 70 °C for 1 h and concentrated under an N_2 stream. Then the mixture was dissolved in *n*-hexane (1 μL) and analyzed by GC-MS. L-Rhamnose, D-xylose and D-glucose were identified by co-injection of the hydrolysate with standard silylated samples. L-Rhamnose, D-xylose and D-glucose were found in **1**, **2** and **3**, giving peaks at 14.02, 16.13, and 28.68 min for **1**, 14.01, 16.13 and 28.62 min for **2**, and 14.01, 16.12 and 28.60 min for **3**, respectively.

Antimicrobial activity

The *in vitro* antibacterial activity tests were evaluated using the microdilution technique against four Gram-negative (*Escherichia coli* ATCC 23999, *Pseudomonas aeroginosa* ATCC 27853, *Salmonella thyphimirium* CCM 5445, *Klebsiella pneumonie* CCM 2318) and four Gram-positive (*Staphylococcus aureus* ATCC 6538-P, *Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC 7064, *Enterococcus faecalis* ATCC 29212) bacterial strains [30]. The bacterial strains were inoculated on Mueller-Hinton broth (Difco) and incubated for 24 h at 37 °C. The inocula were from 24 h broth cultures, and suspensions were adjusted to 0.5 McFarland standards. A series of test tubes were prepared with different concentrations changing from 256 to 0.5 μg mL^{-1} and transferred to the broth in 96-well microtiter plates. Finally, the plates were incubated for 24 h at 37 °C. MIC was the lowest concentration of compound at which microbial growth was inhibited after 24 h. All assays were performed in triplicate. Gentamicin was used as the positive control.

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