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Chemical Constituents of *Campanula lactiflora*

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A new flavonoid glycoside, 4′-O-(p-hydroxybenzoyl)-isorhamnetin-3,7-di-O-β-D-glucopyranoside (**1**), and 4 known compounds, sitosterol β-D-glucoside (**2**), bis(2-ethylhexyl) adipate (**3**), p-hydroxybenzoic acid (**4**) and ethyl docosanoate (**5**), were isolated for the first time from *Campanula lactiflora* and their structures were deduced by high field 1D and 2D NMR and (+) FAB and EI mass spectra.

Key Words: *Campanulaceae*, *Campanula lactiflora*, flavonoid glycoside, steroid, aromatic, ester.

Introduction

The genus Campanula L. belongs to the family Campanulaceae[1]. One of these species, Campanula lactiflora Bieb., is distributed naturally in northern Turkey[1]. Previous phytochemical studies on C. lactiflora have shown the presence of luteolin 7-β-D-glucopyranoside[2], luteolin[3], 3β-acetoxy-lup-20(29)-en-30-al, 3β-acetoxy-ptiloepoxide and 3β-acetoxy-lup-20(29)-ene[4]. In a continuation of our chemical investigation of the chloroform and methanol extracts mixture of air-dried leaves of C. lactiflora, 1 new compound, namely flavonoid glycoside (1), (Fig. 1) and 4 known compounds, namely sitosterol β-D-glucoside[5−6](2), bis(2-ethylhexyl) adipate[6] (3), p-hydroxybenzoic acid[6−8] (4) and ethyl docosanoate[6,9] (5), (Fig. 2) were isolated and characterized by spectral techniques from this plant for the first time. This paper describes the isolation and structure elucidation of a new compound as well as the identification of 4 known constituents from C. lactiflora through spectral analyses.

Experimental

General

NMR spectra were recorded on a Varian NMR at 200 and 400 MHz in $CDCl_3$, CD_3OD, DMSO-d_6 and C_5D_5N. (+) FAB-MS spectrum was recorded on a ZabSpec MS instrument using a glycerol matrix. EI-MS was recorded on a Kratos MS-50 instrument. Melting point was measured on a Kofler hot stage apparatus and is uncorrected. Flash CC was performed on normal (230-400 mesh) and reversed-phase (RP-18) silica

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gel, and PTLC was performed with precoated normal (230-400 mesh) and reversed-phase (RP-18 $F_{254}S$) silica gel.

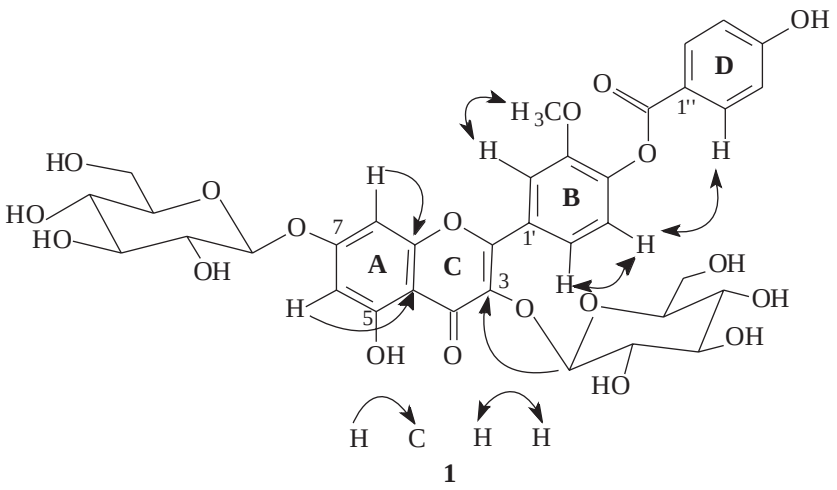

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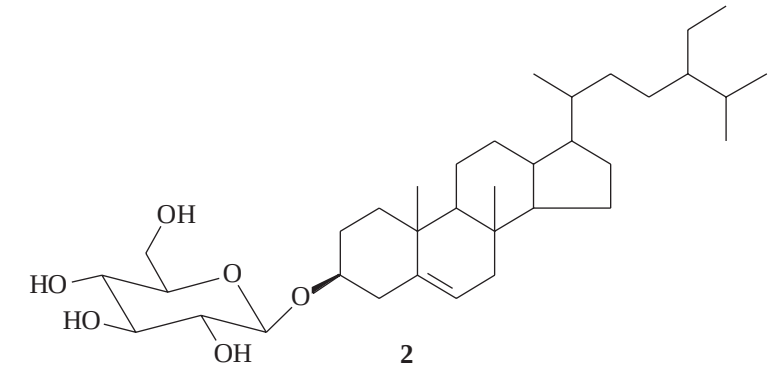
Figure 1. Important HMBC and NOESY correlations of compound **1**.



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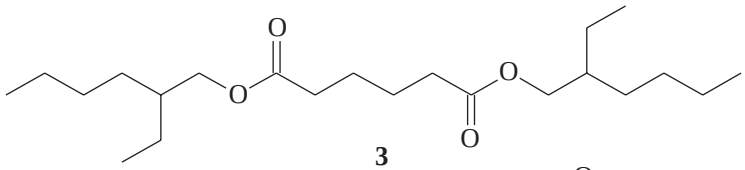

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4

5

Figure 2. Structure of compounds **2-5**.

Plant material

Plant samples were collected in August 1996 at Arpalı, Trabzon Hills (∼2000 m), Turkey. A voucher specimen has been deposited in the Department of Chemistry at Karadeniz Technical University. This species was identified according to the Flora of Turkey[1].

Extraction and Isolation

Air-dried leaves of *C. lactiflora* (388 g) were extracted first with $CHCl_3$ (350 mL, x3, 24 h each) and then with MeOH (300 mL, x3, 24 h each). The $CHCl_3$ and MeOH extracts were concentrated in vacuo at 30-35 ^oC to give brown viscous residues of 4.2 g and 11.6 g wet, respectively. After the TLC analysis, crude mixtures found to be similar were then combined. Crude mixture (10.2 g) was subjected to flash CC on silica gel (300 g, 230-400 mesh). The column was eluted with *n*-hexane (50 mL) followed by a discontinuous gradient with *n*-hexane-$CHCl_3$ (2:1, 150 mL; 1:1, 150 mL), $CHCl_3$ (200 mL), $CHCl_3$-MeOH (10:1, 300 mL; 8:2, 300 mL; 7:3, 300 mL; 1:1, 300 mL), MeOH (200 mL), $CHCl_3$-MeOH-H_2O (7:3:1, 300 mL; 5:5:2, 300 mL) and finally with H_2O (50 mL) to give 37 fractions ($\sim$ 50-60 mL each). After TLC analysis, fractions 1-3 (174 mg) (fatty acid mixture coded CL1), 4 (230 mg) (unidentified terpenes mixtures coded CL2), 5 (322 mg) (terpenes mixtures coded CL3), 6-13 (156 mg) (terpenes mixtures coded CL4), 14-18 (135 mg) (terpenes mixtures coded CL5), 19-22 (235 mg) (terpenes mixtures coded CL6) and 23-37 (670 mg), (unidentified saponins, sugars and flavonoids mixtures coded CL7) were combined.

Fraction CL1 was purified by flash CC on silica gel (20 g, 230-400 mesh). The column was eluted with *n*-hexane (50 mL) followed by a discontinuous gradient with *n*-hexane-$CHCl_3$ (2.5:0.5, 200 mL; 1:1, 60 mL) to give 28 fractions ($\sim$ 5-10 mL each). After TLC analysis, fractions 19-21 gave compound **3** (12 mg, $R_f = 0.72$, in CH_2Cl_2-n-hexane (1:2)). The rest of the fractions were a mixture.

Fraction CL2 was purified by flash CC on silica gel (30 g, 230-400 mesh). The column was eluted with *n*-hexane (50 mL) followed by a discontinuous gradient with *n*-hexane-$CHCl_3$ (2.5:0.5, 200 mL; 1:1, 50 mL) and finally with $CHCl_3$ (50 mL) to give 36 fractions ($\sim$ 5-10 mL each). After TLC analysis, fraction 10-12 were combined and further rechromatographed by flash CC on silica gel (20 g, 230-400 mesh). The column was eluted by a discontinuous gradient with n-hexane-CH_2Cl_2 (2:1, 9 mL; 5:1, 100 mL) to give 35 fractions ($\sim$ 3-5 mL each). After TLC analysis, fractions 19-21 gave compound **5** (9 mg, $R_f = 0.48$, in n-hexane-CH_2Cl_2 (2:1)).

Fraction CL7 (230 mg) was purified by flash CC on silica gel (60 g, 230-400 mesh). The column was eluted with $CHCl_3$ (60 mL) followed by a discontinuous gradient with $CHCl_3$-MeOH (2:0.5, 100 mL; 2:1, 50 mL, 1:1, 50 mL, 1:2, 50 mL), then with MeOH (50 mL) and finally with MeOH-H_2O (10:0.5, 50 mL) to give 33 fractions ($\sim$ 8-10 mL each). After TLC analysis, fractions 7-23 (172 mg) (mixture called CL71), 24-28 (84 mg) (saponin mixture called CL72) and 29-33 (mixture) were combined. CL72 was rechecked by reverse-phase-18 TLC in a MeOH-H_2O (1.5:1) solvent system and found to be a mixture and then rechromatographed by RP-18 (15 g) flash column chromatography (2 $\times$ 60 cm). The column was eluted by a discontinuous gradient with acetone-H_2O (1:1, 20 mL; 1:0.8, 20 mL, 1:0.6, 30 mL, 1:0.5, 30 mL, 1:0.2, 30 mL) and finally with acetone (30 mL) to give 32 fractions ($\sim$5-6 mL each). After TLC analysis, fractions 18-19 (35 mg) were combined and rechromatographed by PTLC (0.5 mm, 20 $\times$ 20 cm, 1 plate) using $CHCl_3$-MeOH (1.5:0.5) to give 3 bands: CL721A: **1**, 7 mg, $R_f = 0.50$ (UV active); CL721B and CL721C contained minor unidentified compounds, $R_f = 0.40$ and $R_f = 0.23$, respectively.

Fraction CL71 (172 mg) was purified by RP-18 (15 g) flash column chromatography (2 $\times$ 60 cm). The column was eluted by a discontinuous gradient with acetone-H_2O (3:1, 30 mL; 3:0.5, 30 mL, 4:0.5 mL) and finally with acetone (30 mL) to give 28 fractions ($\sim$ 5-7 mL each). After TLC analysis, fractions 11-15 gave compound **4** (6 mg, $R_f = 0.65$, in $CHCl_3$-MeOH (3:1)) and fractions 20-21 gave compound **2** (14 mg,

R_f = 0.50, in $CHCl_3$-MeOH (3:1)).

Table. ^1H and ^{13}C NMR spectral data of the compound **1**[a,b] in CD_3OD.

C	^{13}C, δ (ppm)	^1H, δ, (ppm)
2	158.60	-
3	135.42	-
4	179.46	-
5	161.60	-
6	100.15	6.20, s
7	163.80	-
8	94.88	6.40, s
9	159.02	-
10	105.33	-
1'	122.80	-
2'	114.31	7.92, d, (1.6 Hz)
3'	150.82	-
4'	148.40	-
5'	116.06	6.91, d, (8.4 Hz)
6'	123.79	7.60, dd, (1.6, 8.4 Hz)
-OCH_3	56.76	3.92, s
1"	163.04	-
2", 6"	116.07	8.05, 2H, d, (8.8 Hz)
3", 5"	132.26	6.88, 2H, d, (8.8 Hz)
4"	122.77	-
COO	177.12	-
3-O-Glucose		
1	104.10	5.22, d, (7.2 Hz)
2	75.89	3.41[c]
3	78.54	3.42[c]
4	71.45	3.28[d]
5	78.02	3.19[c]
6	62.57	3.52[c]
		3.67[c]
7-O-Glucose		
1	105.23	5.38, d, (7.2 Hz)
2	75.70	3.44[c]
3	78.42	3.42[c]
4	71.32	3.30[d]
5	78.02	3.20[c]
6	62.57	3.52[c]
		3.67[c]

[a]Spectra recorded in CD_3OD.
[b] Assignments based on ^1H, ^{13}C, 2D-COSY, HMQC, HMBC and NOESY spectra.
[c]Signal patterns unclear due to overlapping.
[d] Under CHD_2OD peak (COSY, HMQC).

4′-O-(p-hydroxybenzoyl)-isorhamnetin-3,7-di-O-β-D-glucopyranoside 1: Yellow powder, mp 216^o (dec); $[\alpha]^D$ –12.72^o (CH_3OH, 2 mL; c 5.5 × 10^{-4}); FT-IR (KBr); 3550-3200, 3050-3100, 2950-2800, 1726, 1656, 1650-1450, 1080 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz and DMSO-d_6, 200 MHz: Only one peak

at δ 12.51 ppm (1H, br s, OH-5)) and ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm) see Table; positive FAB-MS (Glycerol) m/z (%); 761(1) $[M+H]^+$, 307(16) [M-2Glc-O–(C_6H_4-OH)-H]$^+$, 285(34) [M-(2Glc-O)-COC_6H_4-OH + 4H]$^+$, 239(23) [243(B ring- O-COC_6H_4-OH)-32-18]$^+$, 228(14) [B ring- O-COC_6H_4-OH – CH_3]$^+$, 202(19) [Glc-O + Na]$^+$, 182(21) [A ring+ C ring + Na]$^+$, 179(22) [Glc-O]$^+$, 163(25) [Glc]$^+$.

Sitosterol 3β-D-glucoside 2: Identified by comparison (^{1}H, ^{13}C, DEPT, COSY) with lit. data[5–6]. ^{1}H NMR (C_5D_5N, 400 MHz) δ (ppm); 5.25 (H-5, under HOD (COSY)), 5.00 (Glc H-1, d, J = 7.6 Hz), 0.94 (3H, d, J = 6.8 Hz), 0.89 (3H, s), 0.85 (3H, t, J = 7.6 Hz), 0.83 (3H, d, J = 6.8 Hz), 0.81 (3H, d, J = 6.8 Hz), 0.62 (3H, s) and ^{13}C NMR (C_5D_5N, 100 MHz) δ (ppm); 140.55 (C), 121.55 (CH), 101.99 (CH), 78.05 (CH), 77.75 (2C, CH), 74.62 (CH), 71.03 (CH), 62.11 (CH_2), 56.46 (CH), 55.83 (CH), 49.94 (CH), 45.61(CH), 42.08 (C), 39.56 (CH_2), 38.86 (CH_2), 37.09 (CH_2), 36.51 (C), 35.95 (CH), 33.78 (CH_2), 31.78 (CH_2), 31.66 (CH), 29.78 (CH_2), 29.06 (CH_2), 28.15 (CH_2), 25.93 (CH_2), 24.12 (CH_2), 22.99 (CH_2), 20.89 (CH_2), 19.61 (CH_3), 19.07 (CH_3), 18.83 (CH_3), 18.64 (CH_3), 11.78 (CH_3), 11.60 (CH_3); EI-MS m/z (%); absent $[M]^+$, 413(16) $[Aglycone]^+$, 396(100) [M-Glucose]$^+$, 164(18) $[Glucose]^+$.

Bis(2-ethylhexyl) adipate 3: Identified by comparison (^{1}H, ^{13}C, DEPT, and COSY) with lit. data[6].^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm); 4.00 (4H, dd, J = 2.8, 5.6 Hz), 2.35 (4H, t, J = 7.2 Hz), 1.69 (4H, t, J = 7.2 Hz), 1.56(m, 4H), 1.38(m, 4H), 1.30(m, 8H), 0.91(t, 12H) and ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm); 173.54 (2C, COO), 66.79 (2C, CH_2), 38.72, (2C, CH_2), 33.99 (2C, CH_2), 30.94 (2C, CH_2), 28.91 (2C, CH_2), 24.47 (2C, CH_2), 23.77 (2C, CH_2), 22.96 (2C, CH_2), 14.05 (2C, CH_2), 10.97 (2C, CH_3); EI-MS m/z (%); 370(7) $[M]^+$.

p-Hydroxybenzoic acid 4: Identified by comparison (^{1}H, ^{13}C, DEPT and COSY) with lit. data[5–7]. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm); 7.86 (2H, d, J = 8.8 Hz), 6.80 (2H, d, J = 8.8 Hz) and ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm); 179.18 (COOH), 163.30 (C), 132.98 (2C, CH), 122.91, (C), 115.99 (2C, CH); EI-MS m/z (%); 138(100) $[M]^+$.

Ethyl docosanoate 5: Identified by comparison (^{1}H, ^{13}C, DEPT and COSY) with lit. data[6,9]. FT-IR; 1725 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm); 4.12 (2H, q, J = 7.2 Hz), 2.28 (2H, t, J = 7.2 Hz), 1.62 (2H, m), 1.27 (36H, m), 0.88 (6H, t, J = 7.2 Hz) and ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm); Ester carbonyl was not detected, 60.13 (O-CH_2), 34.39 (CH_2), 31.91 (CH_2), 29.69 (6C, CH_2), 29.64 (4C, CH_2), 29.58 (CH_2), 29.45 (CH_2), 29.34 (CH_2), 29.25 (CH_2), 29.14 (CH_2), 24.98 (CH_2), 22.68 (CH_2), 14.24 (CH_2), 14.11 ($2CH_3$); EI-MS m/z (%); 368 (6) $[M]^+$, 339 (30) [M-29]$^+$.

Acid Hydrolysis of 1: Compound **1** (2.4 mg) was refluxed with 2N HCl in aq. MeOH (3 mL) for 8 h. The reaction mixture was then concentrated under reduced pressure to remove MeOH. It was then diluted with H_2O (3 mL) and the aqueous layer was adjusted to pH 7 with Ag_2CO_3 and filtered. The supernatant was concentrated and compared with reference sugars and p-hydroxybenzoic acid on TLC (Silica gel, H_2O:MeOH:AcOH:EtOAc, 15:15:20:65). The sugars were detected by spraying the plate with a solution of aniline phthalate in BuOH, which showed that the sugars in **1** were glucose. The p-hydroxybenzoic acid was detected by UV light from the TLC plate.

Results and Discussion

The chloroform and methanol extracts of *C. lactiflora* were repeatedly chromatographed on silica gel to yield compounds **1-5**. By comparing their ^{1}H and ^{13}C NMR signals with reported data[2–19], 1 new compound, namely 4′-O-(p-hydroxybenzoyl)-isorhamnetin-3,7-di-O-β-D-gluco-pyranoside (**1**)[10–17], and 4 known compounds (**2-5**) were identified[5–9].

Compound **1** displayed several strong and broad bands in the range of 1650-1050 cm^{-1} in the FT-IR spectrum, indicative of a flavone skeleton[10–17]. Its molecular formula was determined as $C_{35}H_{36}O_{19}$from the positive FAB-mass spectrum in conjunction with the ^{13}C NMR spectrum.

The ^{1}H NMR spectrum showed the expected signals of 2 aromatic protons singlets at δ 6.20 (1H, bs) and 6.40 (1H, bs) ppm indicating that ring A was disubstituted[12–17] and they were assigned to H-6 and H-8, respectively. Ring B showed a pattern of 3 proton signals at δ 7.92 (1H, d, J = 1.6 Hz), 6.91 (1H, d, J = 8.4 Hz) and 7.60 (1H, dd, J = 1.6, 8.4 Hz), the multiplicity of which showed 1 proton coupled to the remaining 3, which were, in turn, not coupled to each other. The size of the coupling constants (1.6 and 8.4 Hz) is characteristic of *meta* and *ortho* couplings as found in 3′,4′-oxygenated flavonoids[18–19]. In addition, signals in the ^{13}C NMR spectrum at δ 148.40 and 150.82 indicated that the oxygenated carbons are adjacent due to the shielding effect (> 10) each oxygen exerts on its neighboring *ortho* carbons[10,19]. A peak in the ^{1}H NMR spectrum indicated the presence of 1 methoxyl group in the molecule at δ 3.92 appearing as a singlet integrating for 3 protons and in the ^{13}C NMR spectrum as 1 signal at δ 56.76. This methoxyl group was placed on carbon 3′ (148.40) based on HMBC correlations of the methoxyl group with carbon 3′ (Fig. 1). In addition, a cross NOE correlation was seen between $H_{2'}$ and the methoxyl group in the NOESY spectrum. In the ^{1}H NMR spectrum of **1**, the characteristic H_3 signal of a flavone was not exhibited and its ^{13}C NMR spectrum (Table) showed that C-3 was at δ 135.42 ppm, which was in good agreement with C-3-O- glucose flavone[10–17]. In addition, the presence of signals at δ 179.46 ppm was consistent with an α,β-unsaturated carbonyl group in ring C[10–17]. ^{1}H NMR spectra of **1** were also taken in fresh DMSO-d_6 and showed only one hydroxyl peak at δ 12.51 ppm in the downfield region, due to a chelated hydroxyl at C-5, which suggested the presence of a free 5-hydroxyl and substituted 3, 7 and 4′- hydroxyl[15].

There were 2 AX spin systems at δ 8.05 (2H, d, J = 8.8 Hz) and 6.88 (2H, d, J = 8.8 Hz) ppm in the ^{1}H NMR of compound **1**. These chemical shifts were consistent with the p-hydroxybenzoyl group[6,10–12] (Table). The attachment of the p-hydroxybenzoyl unit of **1** to the isorhamnetin 4′-hydroxyl was confirmed by the long-range NOESY correlation between H-5′ (6.91, d, J = 8.4 Hz) and H-2″ (8.05, d, J = 8.8 Hz). All the connections of the components were directly determined by HMBC and NOESY (as shown in the Figure 1). All the data were consistent with those previously reported for similar flavonoids[10–17]. The p-hydroxybenzoyl group was also analyzed on TLC after the acid hydrolysis of compound **1** and was identified as p-hydroxybenzoic acid.

In the ^{1}H NMR spectrum of **1,** two anomeric protons appeared at δ 5.22 (1H, d, J = 7.2 Hz) and 5.38 (1H, d, J = 7.2 Hz) ppm. The J-values of all vicinal couplings in the sugar regions were 7.2 Hz, indicating that the 2 sugars must be β-D glucopyranoside[10,13–17]. These 2 sugars were linked to the aglycone separately, and not as a disaccharide, due to the number of free –OH and chemical data. In the ^{13}C NMR, anomeric carbons of the sugar moieties appeared at δ 104.10 and 105.23 (HMQC, HMBC) (Table). Using 1D (^{1}H, ^{13}C) and 2D NMR (H-COSY, HMQC and HMBC) data individual sugar chemical shifts (δ 3.41-3.67) were

assigned as in the Table. The sugars were also analyzed by TLC after the acid hydrolysis of compound **1** and were identified as glucose.

Based upon the above observations, the structure of compound **1** was established as 4′-O-(p-hydroxybenzoyl)-isorhamnetin-3,7-di-O-β-D-glucopyranoside, a novel natural product isolated from *C. lactiflora*.

Acknowledgments

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