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Secondary Metabolites from the Leaves of *Digitalis viridiflora*

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A new phenylethanoid glycoside, named digiviridifloroside (**1**), was isolated from the leaves of *Digitalis viridiflora* Lindley along with a known phenylethanoid glycoside, calceolarioside A (**2**), two flavonoid glycosides, scutellarein 7-*O*-β-D-glucopyranoside (**3**) and hispidulin 7-*O*-β-D-glucopyranoside (**4**), two cleroindicins, cleroindicins B (**5**) and F (**6**), a nucleoside, adenosine (**7**), as well as a mixture of β-glucopyranosyl-(1→6)-4-*O*-caffeoyl-α/β-glucopyranose and 3,4-dihydroxyphenylethanol. The structure of the new compound was established as 3,4-dihydroxy-β-phenylethoxy-6-*O*-(*E*)-feruloyl-β-glucopyranosyl-(1→6)-4-*O*-(*E*)-caffeoyl-β-glucopyranoside (**1**) based on extensive 1D- and 2D-NMR spectroscopy, as well as HR-ESI-MS. Digiviridifloroside represents a rare type of phenylethanoid glycoside which bears two aromatic acyl units in its structure. In addition to phytochemical studies, the isolates were evaluated for their *in vitro* antimicrobial activities against three pathogenic bacteria and three yeast strains using a microdilution method. Among the tested compounds, **5** exhibited moderate antibacterial activity against *Bacillus cereus* NRRLB 3711 with a MIC value of 25 µg/mL, whereas compounds **5** and **6** showed relatively high anticandidal activity against *Candida* strains with MIC values down to 12.5 µg/mL, in comparison to the standard antimicrobial compounds.

Keywords: *Digitalis viridiflora*, Plantaginaceae, Phenylethanoid glycoside, Digiviridifloroside, Antimicrobial activity.

The genus *Digitalis* (Plantaginaceae) contains biennial or perennial species. It is represented by nine species in the flora of Turkey including *D. viridiflora* Lindley [1]. Previous studies on the genus showed the presence of a wide range of secondary metabolites including phenylethanoid glycosides, cardiac glycosides, steroidal saponins, pregnane glycosides, cleroindicins, flavonoids and anthraquinones [2-6]. In continuation of our systematic survey on the phytochemical composition of *Digitalis* species from Turkey, five phenylethanoid glycosides were recently reported from the initial work on *D. viridiflora* [7]. Further detailed chromatographic studies on the chemical constituents of the leaves of *D. viridiflora* led to the isolation of one new (**1**) and one known phenylethanoid glycoside, two flavonoid glycosides, two cleroindicins and a nucleoside (Figure 1). This paper reports the isolation, structure elucidation and antimicrobial activities of these compounds.

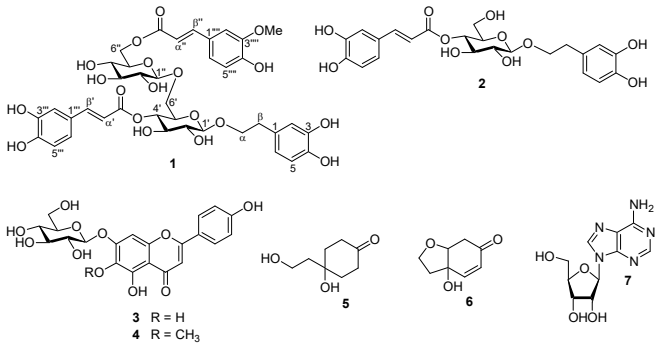

Figure 1: Chemical structures of compounds **1**-**7**.

Compound **1** was obtained as a yellowish amorphous powder. Its UV and IR spectra were characteristic for a phenylethanoid glycoside. It possesses a molecular formula of $C_{39}H_{44}O_{19}$, as determined by the analysis of its HRESIMS (*m/z* 839.2390 [M + Na]$^+$, calcd for $C_{39}H_{44}NaO_{19}$, 839.2375) and ^{13}C NMR data (Table 1). The ^{1}H NMR spectrum (Table 1) of **1** showed resonances at δ_H 7.55 and 6.25 (each d, J = 15.8 Hz) as an *AX* system as well as signals at δ_H 7.00 (d, J = 2.0 Hz), 6.86 (dd, J = 8.1, 2.0 Hz) and 6.74 (d, J = 8.1 Hz) as an *ABX* system attributable to an (*E*)-caffeoyl moiety. Moreover, the spectrum also contained three aromatic signals as an *ABX* system at δ_H 6.70 (d, J = 1.9 Hz), 6.67 (d, J = 8.0 Hz), and 6.55 (dd, J = 8.0 and 1.9 Hz), two geminal benzylic methylene signals at δ_H 2.78 (t, J = 8.0 Hz), and two nonequivalent oxymethylene signals δ_H 4.01 (m) and 3.71 arising from a 3,4-dihydroxyphenylethanol moiety. Furthermore, the presence of two anomeric signals at δ_H 4.37 (d, J = 7.8 Hz) and 4.35 (d, J = 7.7 Hz) revealed the diglycosidic structure of **1**, which was confirmed by the corresponding anomeric carbon resonances at δ_C 105.0 and 104.5 in the ^{13}C NMR spectrum. These findings taken together with 2D NMR experiments (COSY, HSQC and HMBC (Figure 2) revealed the presence of a lugrandoside [2] backbone in **1**. However, the ^{1}H NMR spectrum of **1** contained additional signals at δ_H 7.18 (d, J = 2.0 Hz), 7.05 (dd, J = 8.0, 2.0) and 6.80 (d, J = 8.0 Hz) as an *ABX* type, a pair of *trans*-coupled *AX* type signals at δ_H 7.62 and 6.37 (each d, J = 15.9 Hz) and a methoxy signal at δ_H 3.87 (s) suggesting the presence of a (*E*)-feruloyl unit in the structure of **1**.

The deshielded H_2-6″ (δ_H 4.53 and 4.26) and C-6 ″ (δ_C 64.6) signals of the terminal β-glucopyranose signals indicated that the (*E*)-feruloyl unit was located at C-6″(OH), which was further

Table 1. ^{13}C and ^{1}H NMR data[a] for digiviridifloroside (**1**) (CD_3OD, ^{13}C 125 MHz; ^{1}H 500 MHz).

Position	δ_C (ppm)	δ_H (ppm, *J* in Hz)
Aglycone		
1	131.6	-
2	117.2	6.70 (d, *J* = 1.9)
3	146.2	-
4	144.7	-
5	116.6	6.67 (d, *J* = 8.0)
6	121.5	6.55 (dd, *J* = 8.0, 1.9)
α	72.5	4.01 (m) / 3.71†
β	36.7	2.78 (t, *J* = 8.0)
Glucose		
1′	104.5	4.37 (d, *J* = 7.8)
2′	75.4	3.28 †
3′	75.8	3.62 (t, *J* = 8.0)
4′	72.8	4.83 †
5′	75.1	3.77 (m)
6′	70.3	3.85 †
		3.69 (dd, *J* = 11.5, 4.7)
Glucose		
1″	105.0	4.35 (d, *J* = 7.7)
2″	74.9	3.23 (t, *J* = 8.2)
3″	77.7	3.35†
4‴	77.4	3.33†
5″	75.7	3.48 (m)
6″	64.6	4.53 (dd, *J* = 11.7, 1.8)
		4.26 (dd, *J* = 11.7, 5.8)
Caffeoyl		
1‴	127.8	-
2‴	115.4	7.00 (d, *J* = 2.0)
3‴	146.9	-
4‴	149.8	-
5‴	116.5	6.74 (d, *J* = 8.1)
6‴	123.2	6.86 (dd, *J* = 8.1, 2.0)
α′	114.8	6.25 (d, *J* = 15.8)
β′	147.8	7.55 (d, *J* = 15.8)
C=O	168.7	-
Feruloyl		
1⁗	127.8	-
2⁗	111.8	7.18 (d, *J* = 2.0)
3⁗	150.8	-
4⁗	149.5	-
5⁗	116.8	6.80 (d, *J* = 8.0)
6⁗	124.5	7.05 (dd, *J* = 8.0, 2.0)
α″	115.4	6.37 (d, *J* = 15.9)
β″	147.3	7.62 (d, *J* = 15.9)
C=O	169.2	-
OMe	56.6	3.87 s

[a]Assignments are based on COSY, HSQC and HMBC experiments. † Overlapped.

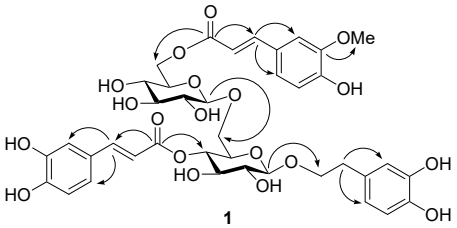

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Figure 2: Key HMBC (C→H) correlations for **1**.

confirmed by the long-range correlation of the carbonyl carbon (δ_C 169.2) of the (*E*)-feruloyl unit with H_2-6″ of the terminal glucopyranose unit in the HMBC spectrum (Figure 2). Based on these spectroscopic data, the structure of **1** was elucidated as 2-(3,4-dihydroxyphenyl)ethyl-*O*-6-*O*-(*E*)-feruloyl-β-glucopyranosyl-(1→6)-4-*O*-(*E*)-caffeoyl-β-glucopyranoside, and named digiviridifloroside.

The known compounds were characterized as calceolarioside A (**2**) [8], scutellarein 7-*O*-β-D-glucopyranoside (**3**) [9], hispidulin 7-*O*-β-D-glucopyranoside (**4**) [10], cleroindicins B (**5**) and F (**6**) [11], and adenosine (**7**) [12] by comparing their spectroscopic data with those published previously. Moreover, a mixture of β-glucopyranosyl-(1→6)-4-*O*-caffeoyl-α/β-glucopyranose and 3,4-dihydroxyphenyl-ethanol, which could be an artefact formed during the isolation procedure, was characterized.

To the best of our knowledge, digiviridifloroside (**1**) is the third example of a rare phenylethanoid glycoside obtained from the genus *Digitalis*, which contains two aromatic acyl units in its structure; the first two such compounds were reported from *D. lanata* [4]. In a very recent study by Skhirtladze *et al.* [13], another new phenylethanoid glycoside esterified with two aromatic acids was reported. Therefore, the occurrence of such rare phenylethanoid glycosides might possess significant chemotaxonomic importance for the genus *Digitalis* within its new family Plantaginaceae.

The *in vitro* antimicrobial activities of the isolates (except for the mixture) were evaluated against three pathogenic bacteria (*Bacillus cereus* NRRLB 3711, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* ATCC 6538) and three yeast (*Candida albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258) strains using a microdilution method. Compounds **4** and **5** displayed moderate activity against *Bacillus cereus* NRRLB 3711 with MIC values 50 and 25 µg/mL respectively, while the rest were inactive against the tested bacteria (Table 2). Compounds **4 - 6** displayed moderate activity against all *Candida* strains tested with MIC values ranging from 12.5 to 100 µg/mL, being **6** the most potent one against *C. parapsilosis* ATCC 22019. To the best of our knowledge the antimicrobial activities of the cleroindicins (**5** and **6**) are being reported for the first time in this study.

Table 2: Antimicrobial activities (MIC, µg/mL) of compounds **1**-**7**.

Comp.	*B. cereus* NRRLB 3711	*P. aeruginosa* ATCC 10145	*S. aureus* ATCC 6538	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 22019	*C. krusei* ATCC 6258
1	>100	>100	>100	>100	>100	100
2	>100	100	>100	>100	>100	100
3	>100	>100	>100	>100	>100	>100
4	50	>100	>100	50	100	50
5	25	>100	>100	25	50	25
6	>100	>100	>100	>100	12.5	25
7	>100	>100	>100	>100	>100	>100
S1	-	-	-	0.031	0.062	0.125
S2	0.002	0.062	0.001	-	-	-

S1: Amphotericin B, S2: Chloramphenicol.

Experimental

General experimental procedures: Optical rotation ($[\alpha]_D^{26}$) was measured on a Perkin-Elmer 341 polarimeter. UV spectra and IR spectra were recorded on a HP Agilent 8453 spectrophotometer and a Perkin-Elmer 2000 FT-IR spectrometer, respectively. NMR experiments were performed on a Bruker Avance DRX 500 instrument. COSY, HSQC and HMBC experiments were run under standard conditions at 300 K, dissolving each sample in 550 µL of 99.8% D CD_3OD (VWR) (^{1}H, δ= 3.34 ppm; ^{13}C, δ= 49.0 ppm). A Q Exactive orbitrap from Thermo Scientific with a HESI ion source was used for HRMS analysis. TLC analyses were carried out on silica gel 60 F_{254} precoated plates (Merck, Darmstadt), and the compounds were stained with 1% vanillin/H_2SO_4 and heating at 105°C. for 1-2 min. For medium-pressure liquid chromatographic (MPLC) separations, Sepacore® Flash Systems X10 / X50 (Büchi) was used with Redi sep columns packed with LiChroprep C_{18} (13, 43 and 130 g, Teledyne Isco) and SiO_2 (40 g, Teledyne Isco). Open column chromatography (CC) was performed using Silica gel 60 (0.063-0.200 mm; Merck, Darmstadt), polyamide and Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA).

Plant material: The leaves of *Digitalis viridiflora* Lindley were collected from Demirköy, Kırklareli, Turkey, in July 2012 and authenticated by Dr. H. Kırmızıbekmez. A voucher specimen (YEF 12012) has been deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey.

Extraction and isolation: The air-dried and powdered leaves of *D. viridiflora* (185 g) were extracted with MeOH (2 L x 2) at 45°C for 4 h. The solvent was evaporated *in vacuo* to afford the crude MeOH extract (45.1 g, yield 24.3%), which was suspended in H_2O (100 mL) and partitioned with $CHCl_3$ (100 mL x 3). The H_2O sub-extract (35 g) was subjected to a polyamide column (120 g) eluting with a gradient solvent system of H_2O/MeOH (100:0 to 0:100) to give 7 main fractions, A-G [7]. Fr. B (12.1 g, eluted with 20% MeOH) was applied to silica gel (150 g) CC eluting with a CH_2Cl_2/MeOH/H_2O gradient (90:10:1 to 50:40:10) to obtain 4 sub-fractions, $B_{1\text{-}4}$. Purification of sub-fraction B_1 (340 mg) by medium pressure liquid chromatography (SiO_2, 40 g) eluting with a stepwise CH_2Cl_2/MeOH gradient (100:0 to 70:30) gave compounds **6** (50 mg) and **5** (7 mg). Repeated chromatography of sub-fraction B_4 (610 mg) by C_{18}-medium pressure liquid chromatography (C_{18}-MPLC, 43 g, using a H_2O/MeOH gradient, 90:10 to 30:70) and Sephadex LH-20 CC (10 g, MeOH), respectively, gave **7** (2 mg). Fraction D (656 mg, eluted with 40% MeOH) was submitted to C_{18}-MPLC (130 g) eluting with a H_2O/MeOH gradient (85:15 to 35:65) to yield a mixture of β-glucopyranosyl-(1→6)-4-*O*-caffeoyl-α/β-glucopyranose and 3,4-dihydroxyphenylethanol (11 mg). Fraction G (380 mg, eluted with 100% MeOH) was applied to Sephadex LH-20 CC (60 g) eluting with MeOH to give sub-fraction G_1 as well as **3** (24 mg). Purification of sub-fraction G_1 (65 mg) by C_{18}-MPLC (13 g) eluting with H_2O/MeOH mixtures (85:15 to 0:100) yielded **2** (4 mg), **4** (4 mg) and **1** (15 mg).

Digiviridifloroside (1)

$[\alpha]_D^{26}$: -59 (*c* 0.1, MeOH).
IR (KBr): 3383, 1698, 1630, 1604, 1515, 1462 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm: 219, 288 (sh), 328.
HR-MS-ESI: *m/z* [M + Na^+] calcd. for $C_{39}H_{44}NaO_{19}$: 839.2375; found: 839.2390.
^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD): Table 1.

Antimicrobial activity assay: A micro-dilution broth susceptibility assay was used, as previously described [14,15]. All microorganisms were stored at -85°C in 15% glycerol prior to the experiments. The bacteria were refreshed on Mueller Hinton agar (MHA, Mast Diagnostics, U.K.), whereas the *Candida* strains were refreshed on Potato Dextrose Agar (PDA, Merck) plates at 37°C. Thereafter, the bacterial suspensions were grown overnight in Mueller-Hinton broth (MHB, Merck, Germany) and were standardized to 1 x 10^8 CFU/mL versus McFarland No: 0.5 in Mueller-Hinton broth (MHB, Merck, Germany), turbidimetrically. Also, *Candida* strains were inoculated, and standardized in the same way, however in sterile saline (% 0.85) to 5 x 10^3 CFU/ per well in RPMI medium (Sigma-Aldrich). Stock solutions of the test samples were prepared in dimethylsulfoxide (DMSO). Dilution series were prepared from 0.6-100 µg/mL accordingly in Mueller Hinton Broth (MHB, Merck) for bacteria and RPMI medium for *Candida* strains in 96-well microtiter plates. Each bacterial (10 µL) and fungal suspension (100 µL) was then added to each well. The last row containing medium with microorganism was used as negative control and medium served as a positive growth control. After incubation at 37°C for 24 h, for staining of viable microorganisms, 0.01% resazurin (20 µL) was added to all of the plates. The first blue well was determined as the minimal inhibitory concentration (MIC, µg/mL). Amphotericin B and chloramphenicol (Sigma, Germany) were used as standard antimicrobial agents at a concentration range of 0.125-64 µg/mL. All experiments were repeated in triplicate and average MICs are given in Table 2.

Supplementary data: HR-MS, ^1H and ^{13}C NMR, COSY, HSQC, HMBC spectra of the new compound **1**.

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