Fitoterapia 134 (2019) 73–80

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

New knowledge about old drugs; a cardenolide type glycoside with cytotoxic effect and unusual secondary metabolites from *Digitalis grandiflora* Miller

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ARTICLE INFO

Keywords:
Digitalis species
Plantaginaceae
cardenolide glycosides
digigrandifloroside
antiproliferative activity

ABSTRACT

Phytochemical investigation of the aerial parts of *Digitalis grandiflora* Miller (Plantaginaceae) led to the isolation of an undescribed cardenolide type glycoside digigrandifloroside (**1**) along with five known compounds, rengyoside A (**2**), rengyoside B (**3**), cleroindicin A (**4**), salidroside (**5**), and cornoside (**6**), from its aqueous fraction of methanolic extract. Structures of the isolated compounds were determined by means of spectroscopic techniques. **1–6** were isolated for the first time from *D. grandiflora*. **2** and **3** are being reported for the first time from *Digitalis* genus and Plantaginaceae family with this study. This is the second report for occurrence of **4** from a *Digitalis* species. Cytotoxic activity of the aqueous fraction was also tested against HEp-2 (Human larynx epidermoid carcinoma) and HepG2 (Human hepatocellular carcinoma) cancer cell lines and L929 (Mouse fibroblast cell) non-cancerous cell line. Aqueous fraction showed stronger cytotoxicity on HEp-2 cells than HepG2. Therefore, the cytotoxic activity of **1**, **2**, **4,** and **6** were tested against HEp-2 and L929 cell lines. **3** and **5** couldn't be tested due to their insufficient amount. **1** showed the highest cytotoxicity against HEp-2 cells with IC_{50} value 10.1 μM when compared with the positive control, etoposide and **2–6** (IC_{50} of etoposide; 39.5 μM).

1. Introduction

Plants have been used as folk medicines for a long time in history for the therapy of a wide range health problems. Evaluation of anticancer agents used in therapy, from 1940s to 2011, have shown that from 175 small molecular compounds approved 85 of them are natural products or derived from natural sources [23].

Although, there is no traditional use of genus *Digitalis* L., its first known use as a medicine was in 18th century by Withering. Nearly more than a half-century *Digitalis* species and *Digitalis*-derived cardioactive glycosides have been investigated for their effects in heart failure. Their toxicities and therapeutic effects were identified in detailed [18].

In the flora of Turkey, the genus *Digitalis* is presented by 9 species, 5 of which are endemic [5]. *Digitalis* species was formerly a member of Scrophulariaceae family, but after phylogenetic and chemotaxonomic studies it has been moved to Plantaginaceae. Cardioactive glycosides, phenylethanoid glycosides, flavonoids, and anthraquinones were reported from the genus *Digitalis* so far [16–18,27].

Cardioactive glycosides show their effect on myocardial cells by binding to Na-K ATPase pump to inhibit this ion transport enzyme [15]. Recent studies showed the role of cardioactive glycosides in cancer prevention and anticancer agents [22]. It was shown that Na-K ATPase pump α-1 subunit can play a role in proliferation and migration in cancer cells. The results of the study indicated that in cancer cells there became an over expression of α-1 subunit compared to normal cells. So, targeting this subunit could be an option in cancer therapy [22]. Some other reports also suggested different mechanisms for the cytotoxicity. Apoptosis, autophagy and immunologic cell death were also reported for cardioactive glycosides [30]. There are several reports about *in vitro* cytotoxic activity of cardioactive glycosides against different human cancer cell lines [27,28]. Fujino et al. [7] pointed out the selectivity between cancer cells and non-cancerous cells in their report. A cardioactive glycoside glucodigifucoside showed selective high cytotoxicity against ACHN (human renal adenocarcinoma cell line) when compared with HK-2 (normal human renal proximal tubule-derived cell line) [7].

Digitalis species also reported to have emetic, antibacterial, antiviral, antifungal, antiinflammatory and antioxidant activities [3,11,21,24,25,33].

The purpose of our study is the isolation of secondary metabolites with cytotoxic activity from the aerial part of *D. grandiflora*. *D. grandiflora* is an annual or perennial herb growing wildly in north-west

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https://doi.org/10.1016/j.fitote.2019.02.001
Received 6 December 2018; Received in revised form 2 February 2019; Accepted 3 February 2019
Available online 06 February 2019
0367-326X/

Turkey and Balkan region. There are only a few studies about the phytochemical content of the plant so far. Therefore, to clarify the chemical content, phytochemical studies were planned to perform. Bioactivity tests were also carried out according to the recent research interest on *Digitalis* species and cardenolide type glycosides. In this study, cytotoxicity was tested against both cancer (HEp-2, HepG2) and non-cancerous (L929) cell lines to determine the selectivity. This selectivity is important for the compounds to become an anticancer drug candidate.

2. Experimental

2.1. General experimental procedures

Optical rotations were recorded on a JASCO P-2100 polarimeter. HR-ESIMS data were obtained from Bruker Daltonics APEX III. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz) and 2D-NMR spectra of the compounds were measured in CD_3OD with an Agilent Varian VNS500 spectrometer. Chemical shifts (ppm) were referenced to the residual solvent peaks (δ_H 3.31 and δ_C 49.0). Tetramethylsilane was used as internal standard.

2.2. Chemicals/reagents and cell culture material

Column chromatography separations were carried out with polyamide (Polyamide 6, 50–160 μm; obtained from Sigma-Aldrich; St. Louis, MI, USA), silica gel (Kieselgel 60, 70–230 mesh, 0.063–0.2 μm, from Merck; Darmstadt, Germany) and RP silica gel (LiChroprep C18, 40–63 μm, Merck). D-glucose and L-rhamnose were obtained from Sigma-Aldrich. Lanatoside C was purchased from Fluka AG. TLC plates Silica gel 60 F_{254}, RP18 F_{254} and all solvents were obtained from Merck. Etoposide was purchased from Deva Holding A.S. (Istanbul, Turkey). Fetal bovine serum (FBS), minimal essential medium with Earle's salts (MEM-Earle) and antibiotics (penicillin and streptomycin) were obtained from Biochrom AG (Berlin, Germany). Trypsin/EDTA solution was purchased from Merck. Dulbecco's Modified Eagle's Medium (DMEM), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and sodium dodecyl sulphate was purchased from Sigma (St Louis, MI, USA). HEp-2 (Human larynx epidermoid carcinoma) cell line was obtained from Riken Bioresource Center Cell Bank (Tokyo, Japan). HepG2 (Human hepatocelular carcinoma) cells was obtained from Health Science Research Resources Bank (Osaka, Japan). L929 (Mouse fibroblast cell) cell line was purchased from Biota Lab (Istanbul, Turkey).

2.3. Plant material

Digitalis grandiflora Miller was collected from Kırklareli, Turkey in July, 2013. Identification of the plant was done by Dr. Z. Ceren Arituluk (Hacettepe University Faculty of Pharmacy Department of Pharmaceutical Botany, Ankara, Turkey). Voucher specimen was deposited in Hacettepe University Faculty of Pharmacy Herbarium (HUEF13007).

2.4. Extraction and isolation

Aerial parts of *D. grandiflora* was dried in appropriate conditions. Dried plant material was powdered, 290 g out of it was weighed and macerated in 5 L MeOH overnight, then extracted for 8 h at 40 °C. This procedure was repeated three more times. The extracts were combined and dried by evaporating under vacuum at 40 °C. The methanol extract was lyophilized to yield 37 g dry weight. The lyophilized extract was dissolved in 400 mL of water and partitioned with petroleum ether to discard lipophylic fractions. Aquoeus extracts (26 g) was used for cytotoxic activity tests and isolation studies.

The aquoeus extract (25 g) was subjected to polyamide column chromatography and eluted with H_2O and continued by a gradient system with increasing ratios of MeOH from 25 to 100% MeOH to yield five different fractions (Fr. A, 21.3 g; Fr. B, 1.8 g; Fr. C, 0.50 g; Fr. D, 0.20 g; Fr. E, 0.10 g). Further chromatographic methods applied on Fr. A, which is the most cytotoxic fraction among the all fractions, to yield **1–6.** Fr.A was dissolved in H_2O and liquid-liquid extraction was performed using *n*-BuOH. 1 g of *n*-BuOH fraction (total 8.2 g) was subjected to silica gel column chromatography with a stepwise gradient system of $CHCl_3$: MeOH: H_2O (100:0:0–50:50:5). Isolation studies on subfractions yield to **1–6**.

1 and **5** were isolated from the silicagel column subfraction obtained by $CHCl_3$: MeOH: H_2O (75:25:2.5). Subfraction (65 mg) was applied to vacum liquid chromatography and eluted with MeOH: H_2O. **5** (6.5 mg) was eluted with 16% MeOH, **1** (9.4 mg) was eluted with 45% MeOH.

2 were isolated from the silicagel column subfraction obtained by $CHCl_3$: MeOH: H_2O (65:35:3.5). Subfraction (109 mg) was applied to vacum liquid chromatography and eluted with MeOH: H_2O. **2** (8.7 mg) was eluted with 8% MeOH.

3 and **6** were isolated from the silicagel column subfraction obtained by $CHCl_3$: MeOH: H_2O (70:30:3). Subfraction (140 mg) was applied to vacum liquid chromatography and eluted with MeOH: H_2O. Elution with 10% MeOH **3** (5.8 mg) and **6** (7 mg) were isolated.

4 was isolated from the silicagel column subfraction obtained by $CHCl_3$: MeOH: H_2O (80:20:2). Subfraction (169 mg) was applied to sephadex column chromatography and then applied to vacum liquid chromatography and eluted with MeOH: H_2O. **4** (99.2 mg) was eluted with 5% MeOH.

2.5. Cytotoxic activity

HEp-2, HepG2 and L929 cells were used for cytotoxic activity tests. Cells were seeded in a 96-well plate at a density of 1×10^5cells/mL for HEp-2, 4×10^5 cells/mL for HepG2 and 8×10^4 cells/mL for L929 cells. MEM's Earle medium for HEp-2 and L929 cells and Dulbecco's Modified Eagle's Medium (DMEM) for HepG2 cell were used.

Cells were cultured in respective culture media supplemented with 10% FBS, 1% penicillin-streptomycin solution in a humidified 5% CO_2 in air at 37 °C for 24 h. Cells were treated with different concentrations of samples for the next 48 h. After incubation, the cells were washed and replaced by fresh medium. 10 μL of MTT solution (5 mg/mL in PBS) was added and incubated for 4 h. After that, 100 μL of 10% SDS (sodium dodecyl sulphate) was added to each well to dissolve formazan crystals. The absorbance was measured at 570/620 nm using microplate reader. Results were given as percentage of inhibitory effect treated cells to untreated cells that served as control [29]. Three independent test results were considered, averages and standard deviations were calculated and shown in the figures.

2.6. Statistical analysis

Results of MTT experiments are expressed as mean ± standard deviation. The statistical significance was determined by one- way ANOVA post hoc Dunnett's test by using IBM SPSS Statistic 23 software. The IC_{50} values were computed by GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA).

2.7. Digigrandifloroside (1) (12-epidigoxigenin 3-O-β-D-glucopyranosyl (1 → 4) β-D-digitoxopyranoside)

White amorphous powder; $[\alpha]_D^{22}$ + 7.0 (c 1.0, MeOH); ^{1}H and ^{13}C NMR data, see Tables 1–3; HR-ESIMS: 705.34919 *m/z* $[M + Na]^+$ (calculated for $C_{35}H_{54}NaO_{13}$ 705.34621).

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data and HMBC correlations of **1** in CD_3OD.

Position	Multiplicity	δ_C ppm	δ_H ppm	*J* (Hz)	HMBC (H → C)
Aglycon					
1	CH_2	31.3	1.38–1.52[b]		C-2, C-5, C-10
2	CH_2	27.5	1.59–1.70[b]		C-1
3	CH	74.5	4.02 brs		C-5
4	CH_2	31.0	1.29[b]		C-5
			1.44–1.52[b]		
5	CH	38.0	1.65[b]		C-4, C-7
6	CH_2	27.8	1.26[b]		C-4, C-5, C-8
			1.90[b]		
7	CH_2	22.5	1.32[b]		C-5
			1.77[b]		C-8
8	CH	42.8	1.63[b]		C-11, C-14
9	CH	30.2[a]	2.12 m	(10.0)	C-14
10	C	36.0			
11	CH_2	30.3[a]	1.55[b]		C-8
12	CH	76.5	3.59 brs		C-9, C-11, C-14, C-18
13	C	54.6			
14	C	86.6			
15	CH_2	35.0	1.65[b]		C-13, C-14, C-17
			2.27 m		C-16
16	CH_2	30.2[a]	1.82		
			2.06 m		C-14
17	CH	46.7	3.39[b]		C-22
18	CH_3	17.7	0.83 s		C-12, C-13, C-14, C-17
19	CH_3	24.2	0.94 s		C-5, C-10,
20	C	179.6			
21	CH_2	75.7	4.94 d	(18.5)	C-22
			4.98 d	(18.5)	
22	CH	117.7	5.90 s		C-17, C-21
23	C=O	177.3			
Digitoxose					
1′	CH	96.8	4.92 brd	(8.0)	C-3, C-2′, C-3′
2′	CH_2	39.1	1.74[b]		C-1′
			1.95[b]		C-4′
3′	CH	68.6	4.30 ddd	(3.0/3.0/3.0)	C-1′
4′	CH	84.3	3.28[b]		C-3′, C-1″
5′	CH	69.6	3.87		C-4’
6′	CH_3	18.6	1.30 d	(6.5)	
Glucose					
1″	CH	105.8	4.38 d	(8.0)	C-4′, C-2″
2″	CH	75.1	3.23 dd	(8.0/8.0)	C-3″
3″	CH	77.9	3.34[b]		C-1″
4″	CH	71.1	3.34[b]		C-3″, C-5″
5″	CH	77.7	3.28[b]		
6″	CH_2	62.3	3.70 dd	(12.0/5.0)	C-4″
			3.82 dd	(12.0/5.0)	

[a] These signals are exchangeable.
[b] Signal coupling patterns are unclear due to overlapping.

2.8. Acid hydrolysis and configuration determination of sugars

Compound **1** (2 mg) was dissolved in 1 mL of methanol and hydrolyzed with 2 mL of 12% HCl at 80 °C. The mixture was extracted with 2 mL of chloroform twice. The aqueous layer was dried under vacuum. Residue was dissolved in 100 μL pyridine and 100 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was added for derivatisation. D-glucose was used as sugar standard. D-digitoxose was obtained by hydrolyzation of the known compound lanatoside C. The same acid hydrolysis procedure was applied to lanatoside C. Sugar standards were also dissolved in 100 μL pyridine and 100 μL of MSTFA was added for silylation. Samples were analyzed by GC/MS analysis. Agilent 7890b Gas Chromatograph was used with HP-5 ms column (30μmx250μmx0.25 μm). The injection volume was 1 μL. Samples were applied to the column held initially at 40 °C for 1 min and then increased to 260 °C with a 15 °C/min heating ramp. The retention times (min) of the derivatized sugar standards were as follows: D-glucose (14.23), D-digitoxose (13.59). D-glucose and D-digitoxose were detected in compound **1** by comparison with retention times of standard sugars.

Table 2
^{13}C NMR (125 Hz) data of isolated compounds **2–6** in CD_3OD.

	2	**3**	**4**	**5**	**6**
Aglycon	δ_C	δ_C	δ_C	δ_C	δ_C
1	70.5	70.9		130.7	69.3
2	36.2	38.5	59.1	130.9	154.5
3	31.3	38.4	45.6	116.1	127.9
4	70.8	215.5	70.8	156.7	187.9
5	31.3	38.4	36.0	116.1	127.9
6	36.0	38.5	31.3	130.9	154.4
7			70.8		
8			31.3		
9			36.0		
α	67.0	67.4		71.6	65.7
β	43.0	42.5		36.3	41.0
Glucose					
1″	104.4	104.9		104.3	104.3
2′	75.1	75.6		75.1	75.0
3′	78.1	78.7		78.1	78.1
4′	71.6	72.2		72.1	71.6
5′	78.0	78.5		77.9	77.9
6′	62.8	63.3		62.7	62.7

3. Results and discussion

3.1. Structure elucidation of compounds

Isolation studies carried out on aqueous fraction of methanolic extract of *D. grandiflora* afforded 6 pure compounds (**1–6**), one of which was a new compound (**1**). Their chemical structures are shown in Fig. 1.

1, was obtained as a white amorphous powder, with the molecular formula $C_{35}H_{54}O_{13}$ was established by HR-ESIMS *m/z* 705.34919 $[M + Na]^+$ in association with 35 carbon signals observed at ^{13}C NMR data. The ^{13}C NMR and DEPT spectra revealed 35 carbon signals including three methyl (δ_C 17.7, 18.6, 24.2; CH_3), eleven methylene (δ_C 22.5, 27.5, 27.8, 30.2, 30.3, 31.0, 31.3, 35.0, 39.1, 62.3, 75.7; CH_2), sixteen methine (δ_C 30.2, 38.0, 42.8, 46.7, 68.6, 69.6, 71.1, 74.5, 75.1, 76.5, 77.7, 77.9, 84.3, 96.8, 105.8, 117.7; CH), and five quaternary (δ_C 36.0, 54.6, 86.6, 177.3, 179.6; C) carbons (Table 1). The detailed examination of HR-ESI-mass (MS), ^{1}H, ^{13}C, COSY, HMQC, HMBC, and NOESY NMR spectra suggested this substance should be a cardenolide type glycoside. All ^{1}H and ^{13}C NMR data and HMBC correlations were given in Table 1. ^{1}H NMR signals of *α,β*-unsaturated γ-lactone [δ 4.98 (1H, brd, J = 18.5 Hz, H-21b), 4.94 (1H, brd, J = 18.5 Hz, H-21a) oxymethylene signals, and 5.90 (1H, brs, H-22) olefinic signal] have been seen at ^{1}H NMR spectrum. At ^{13}C NMR spectrum the signals corresponding to these protons [δ 117.7, CH, (C-22) and 75.7, CH_2, (C-21)] with C signals [δ 177.3, CO, (C-23) and 179.6 C, (C-20)] supported the presence of a lactone ring in the structure. Two methyl signals at δ 0.83 (3H, s, H-18) and δ 0.94 (3H, s, H-19) and an oxygen bearing quaternary carbon (δ 86.6, C-14) was determined. Signals of methyl and methylene protons between δ_H 1.30–2.30 ppm were evaluated together with the data given above. Oxymethylene signals at δ_H 4.02 (brs, H-3) and 3.59 (brs, H-12) ppm were also detected. The ^{13}C signals corresponding to these protons were determined using HMQC spectra. Fragments were generated by means of ^{1}H-^{1}H COSY spectrum and they were coupled up by using long range correlations observed in HMBC spectrum. According to these findings, the aglycone of **1** was considered to possess similar chemical structure with digoxigenin. Therefore, ^{1}H and ^{13}C NMR spectral data was compared with the data of digoxigenin [9,20,26] and it was recognized that the data of **1** showed discrepancy

Table 3
^{1}H NMR (500 MHz) data of isolated compounds **2–6** in CD_3OD.

	2		**3**		**4**		**5**		**6**	
Aglyco	δ_H ppm	*J* (Hz)	δ_H ppm	*J* (Hz)	δ_H ppm	*J* (Hz)	δ_H ppm	*J* (Hz)	δ_H ppm	*J* (Hz)
2	1.41 t	(12.5)	2.03 m		3.74 t	(7.0)	7.06 d	(8.5)	7.02 d	(10.0)
	1.72 m		1.85 m							
3	1.66 m		2.68 m	(14.5)	1.70[a]		6.69 d	(8.5)	6.12dd	(10.0/2.5)
			2.20 d							
4	3.52 m									
5	1.66 m		2.68 m	(14.5)	1.43 td	(12.0/4.0)	6.69 d	(8.5)	6.12dd	(10.0/2.5)
			2.20 d		1.73[a]					
6	1.41 t	(12.5)	2.03 m		1.64[a]		7.06 d	(8.5)	7.02 d	(10.0)
	1.72 m		1.85 m							
7					3.52 m					
8					1.64[a]					
9					1.43 td 1.73[a]	(12.0/4.0)				
α	4.08 m		4.13 m				4.03 m		3.99 m	
	3.71 m		3.77 m				3.70 m		3.62 m	
β	1.77 t	(7.5)	1.90 t	(7.0)			2.83 m		2.05 t	(6.5)
Glucose										
1′	4.25 d	(7.5)	4.28 d	(7.5)			4.29 d	(8.0)	4.22 d	(7.5)
2′	3.15 dd	(7.5/8.5)	3.16 dd	(7.5/9.0)			3.18 dd	(8.5/8.0)	3.14 dd	(7.5/9.0)
3′	3.34 [a]		3.35[a]				3.35 t	(8.5)	3.34 t	(9.0)
4′	3.27 [a]		3.32[a]				3.30[a]		3.28 dd	(7.5/9.0)
5′	3.26 m		3.31[a]				3.28 m		3.26 m	
6′	3.86 dd	(11.5)	3.88 brd	(12.0)			3.86 brd	(12.0)	3.85 brd	(12)
	3.65 d	(11.5/5.0)	3.66 brd	(12.0)			3.66 dd	(12.0/5.0)	3.66 dd	(12/5.5)

[a] Signal patterns are unclear due to overlapping.

from the literature. The ^{13}C signal belongs to C-18 methyl group (δ 17.7, CH_3) at C-13 of **1** shifted downfield approximately 8 ppm and signal of C-13 (δ 54.6, C) shifted downfield approx. 3 ppm [20,26] (Table 1). The relative configuration of these groups was clarified by the correlations observed in the NOESY spectrum. The key NOESY correlations were shown in Fig. 2. The NOESY correlations were observed between H-12 with H-18 in the spectrum. In addition, there was no correlation observed between H-17 and H-18. These findings suggested that the C-12 hydroxyl group should be attached to the skeleton in α configuration which differs from digoxigenin. The ^{13}C NMR data of 12- α hydroxyl and 12- β hydroxyl digoxigenin given in the literature were compared with the data of compound **1** [9]. The chemical shifts observed for C and D rings confirmed that hydroxyl group at C-12 is in α configuration. ^{13}C NMR spectra was compared with the literature data and A/B and C/D connections were found as *cis* and B/C was *trans*. With all the data mentioned above showed that aglycone was a 12 epimer of digoxigenin.

Anomeric proton signals at δ 4.92 (H, brd, J = 8.0 Hz, H-1′) and 4.38 (H, d, J = 8.0 Hz, H-1″) at ^{1}H NMR spectrum indicated the presence of a sugar moiety with two sugar unites. The coupling constants of these anomeric signals explained that both sugar units are in β-configuration. The signals: a methyl group at δ 1.30 (3H, d, J = 6.5 Hz, H-6′); seven oxymethines at δ 3.23 (H, dd, J = 8.0, 8.0 Hz, H-2″), 3.28 (H, m, H-4′), 3.28 (H, m, H-5″), 3.34 (H, m, H-3″), 3.34 (H, m, H-4″), 3.87 (H, m, H-5′), 4.30 (H, ddd, J = 3.0, 3.0, 3.0 Hz, H-3′); a methylene at δ 1.74 (H, m, H-2'a) and 1.95 (H, m, H-2'b), an oxymethylene at δ 3.70 (H, dd, J = 12.0, 5.0 Hz, H-6″a) and 3.82 (H, dd, J = 12.0, 2.0 Hz, H-6″b). The sugar moiety was deduced as digitoxose and glucose using ^{1}H-^{1}H COSY spectrum and carbon signals were identified using HMQC correlations. The signals at δ_H 1.74–1.95 ppm (H-2′) and δ_H 1.30 ppm (3H, d, J = 6.5 Hz, H-6′) showed the 2,6 dideoxyose structure of digitoxose as the first sugar. The long range correlations observed between H-1″ of glucose (δ_H 4.38, d) and C-4′ of digitoxose (δ_C 84.3) in the HMBC spectrum indicated that the second sugar unite was attached at the 4th position of the first sugar unite (Fig. 3, Table 1). Combining the HMBC and COSY spectra the sugar moiety was identified as β- glucopyranosil (1 → 4) β- digitoxopyranoside. HMBC correlation between H-1′ and C-3, and the occurrence of H-3 oxymethine signal at δ 4.02 as a broad singlet explained that the sugar unit connected to the aglycone at C-3 in β-orientation. Configuration of the sugars were determined as D-glucose and D-digitoxose by GC/MS analysis. Therefore, the structure of **1** was determined to be 12-epidigoxigenin 3-O- β-D-glucopyranosyl (1 → 4) β-D-digitoxopyranoside and named as digigrandifloroside. There is no literature data that the presence of 12-epidigoxigenin as a naturally occuring aglycone in the cardenolide type glycosides so far. Synthetic derivatives of 12-epidigoxigenin were reported in the literature [9]. Therefore, we have suggested this unusual aglycone was isolated from nature for the first time with this study.

The known compounds were characterized as rengyoside A (**2**), rengyoside B (**3**) [31], cleroindicin A (**4**) [32], salidroside (**5**) and cornoside (**6**) [19] comparing their NMR and physical data with published data in the literature. ^{1}H and ^{13}C NMR data of the known compounds were given in Tables 2 and 3. Rengyosides A and B are being reported for the first time from *Digitalis* species and Plantaginaceae family with this study. This is the second report for the isolation of cleroindicin A from *Digitalis* species, the first report was from *D. trojana* [17].

3.2. Cytotoxic activity

The aqueous fraction of *D. grandiflora* methanolic extract was tested for its cytotoxicity against Hep-2, HepG2 cancer cells and L929 noncancerous cells. Extract showed higher cytotoxicity with an IC_{50} value of 63.7 μg/mL on Hep2 cells when compared to HepG2 cells with an IC_{50} value of 514 μg/mL (Table 4). This difference showed that the extract has selectivity between cancer cell lines. Therefore, Hep2 cells were selected for the cytotoxicity tests of the isolated compounds. Cytotoxic activity of the extract was found to be lower on L929 cell line with an IC_{50} value of 215 μg/mL (Table 4). The selectivity was also observed between cancer and non-cancerous cell lines.

Digigrandifloroside (**1**), rengyoside A (**2**), cleroindicin A (**4**), and cornoside (**6**) were evaluated for their cytotoxic activity against HEp-2 cancer cell line and L929 noncancerous cell line. Etoposide was used as positive control as it is a semisynthetic derivative of a nature originated compound, podophyllotoxin. IC_{50} values for rengyoside A (**2**), cleroindicin A (**4**) and cornoside (**6**) on HEp-2 cell line were found to be

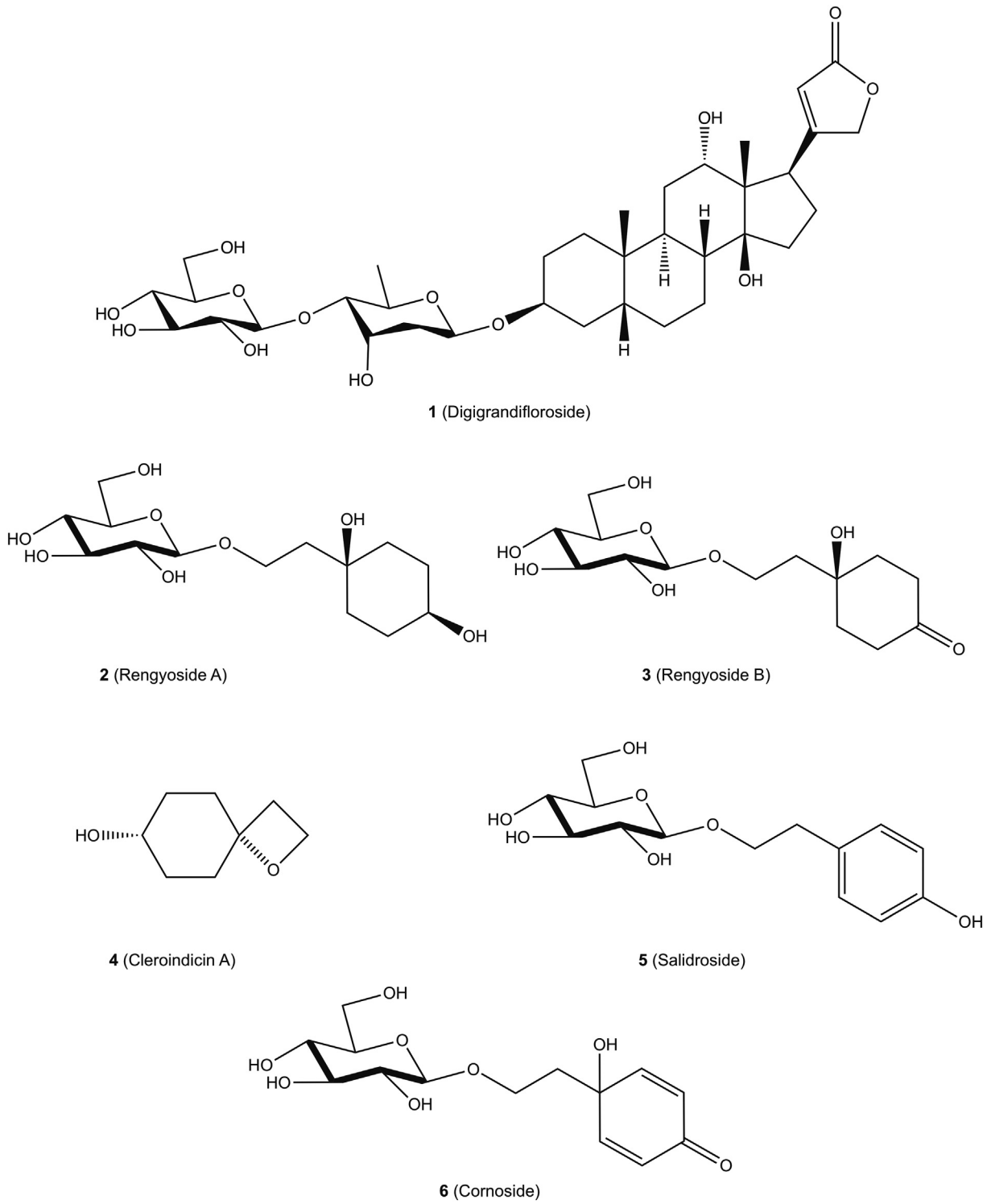

Fig. 1. Structures of the isolated compounds from *D. grandiflora*

382, 2.56×10^3, 2.03×10^3 μM respectively. These compounds showed concentration dependent low cytotoxicity against selected cancer cell line. Their cytotoxicity on L929 cells were almost two folds lower than they had on HEp-2 cells. IC_{50} values for rengyoside A (**2**) and cornoside (**6**) on L929 cell line were found to be 1.20×10^3 and 3.77×10^3 μM, respectively (Table 4).

The new compound digigrandifloroside (**1**) showed concentration dependent high cytotoxicity against HEp-2 cells which is significant when compared with the positive control etoposide (Fig. 4, Table 4). Digigrandifloroside has an IC_{50} value of 10.1 μM on HEp-2 cells and IC_{50} value of 12.9 μM on L929 cells (Table 4). In comparison with an anticancer agent etoposide, digigrandifloroside showed higher potential on HEp-2 cell line. In previous reports cardenolide type glycosides were tested on several cell lines. Digoxin was tested against brain SH-SY5Y, brain SK-N-AS, breast MDA-MB-231, breast MDA-MB-435, liver Hep3B, prostate PC3, prostate PPC-1, and transformed human B-lymphocytes P493-Myc cancer cells [4]. SH-SY5Y and SK-N-AS neuroblastoma cell lines were found to be the most sensitive among the tested

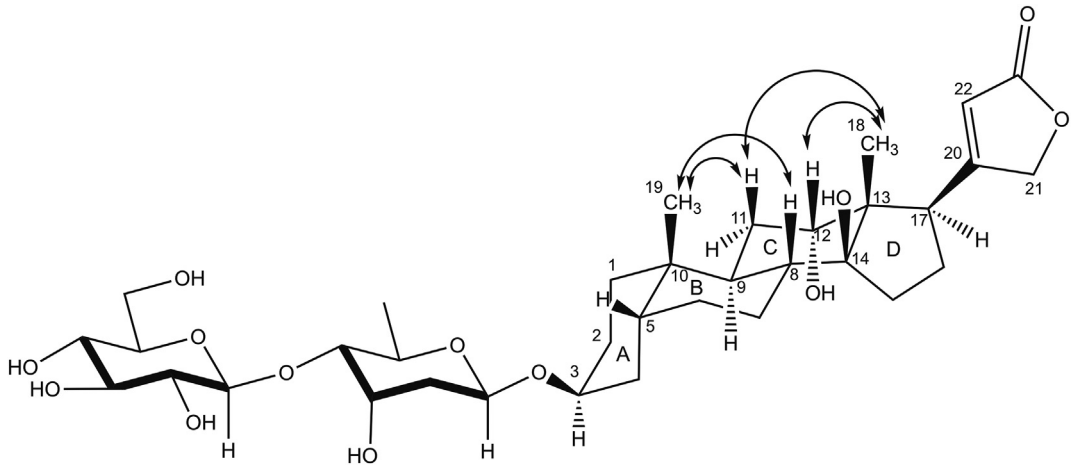

Fig. 2. The key NOESY correlations of compound 1

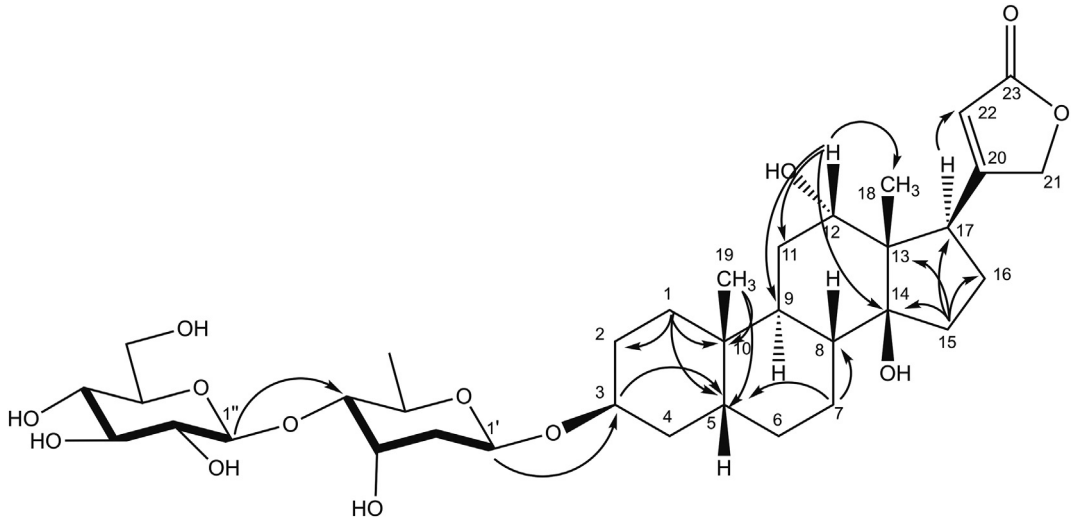

Fig. 3. The key HMBC correlations of compound **1**

Table 4
IC_{50} values of extract and compounds against HEp-2, HepG2 and L929 cell lines

Extract/Compounds	IC_{50} values		
	Cell lines		
	Hep-2	HepG2	L929
D. grandiflora extract	63.7 µg/mL	514 µg/mL	215 µg/mL
Digigrandifloroside **(1)**	10.1 µM	nd.[a]	12.9 µM
Rengyoside A **(2)**	382 µM	nd.[a]	1.20×10^3 µM
Cleroindicin A **(4)**	2.56×10^3 µM	nd.[a]	2.58×10^3 µM
Cornoside **(6)**	2.03×10^3 µM	nd.[a]	3.77×10^3 µM
Etoposide	39.5 µM	nd [a]	45.2 µM

[a] Not determined.

cell lines with the IC_{50} values of 34 and 22 ng/mL. The clinical studies performed on digoxin at prostate cancer is in Phase II level [1]. Lanatoside C was tested against U87 glioblastoma cells and the IC_{50} value was calculated as 1 µg/mL [2].

Even there is no other study reported on HEp-2 cancer cells treated with cardenolide glycoside so far, digigrandifloroside showed lower cytotoxicity than digoxin and some other cardenolide glycosides against cancer cells. In this study, IC_{50} of digigrandifloroside was found as 10.1 µM corresponding to 6.9 µg/mL. The epimerisation at C-12 position could be responsible from the lower cytotoxic activity of digigrandifloroside when compared with other cardenolide glycosides.

When the cytotoxicity results evaluated in detailed, it could be seen that digigrandifloroside showed biphasic effect, both cytotoxic and cytostatic (antiproliferative), depends on concentration, on L929 cell line (Figs. 5 and 6). A dramatical decrease in cell viability (cytotoxic effect) observed till 5 µg/mL concentration corresponding to 7.33 µM (cell viability; 52.51%). Cytostatic (antiproliferative) effect was observed with the increase of digigrandifloroside concentration. Even at 100 and 200 µg/mL concentrations corresonding to 146 and 293 µM of digigrandifloroside, viability of L929 cells were found as 41.0 and 36.4% respectively (Fig. 5). However, the viability % of HEp-2 cells at 100 and 200 µg/mL concentrations corresponding to 146 and 293 µM of digigrandifloroside, were found as 1.23 and 1.09% respectively (Fig. 4). These results showed that the cytostatic activity can't be seen on Hep-2 cells, as high cytotoxicity have been detected. Although the IC_{50} values of digigrandifloroside were found to be similar on Hep-2 and L929 cells, it has a type of activity selectivity between cancer and noncancerous cells (Fig. 6).

4. Conclusion

Studies on aqueous fraction of methanolic extract of *D. grandiflora* afforded 1 new total 6 compounds. All compounds were isolated for the first time from *D. grandiflora*. Among the isolated compounds, rengyosides A (**2**) and B (**3**) were reported in *Digitalis* genus and

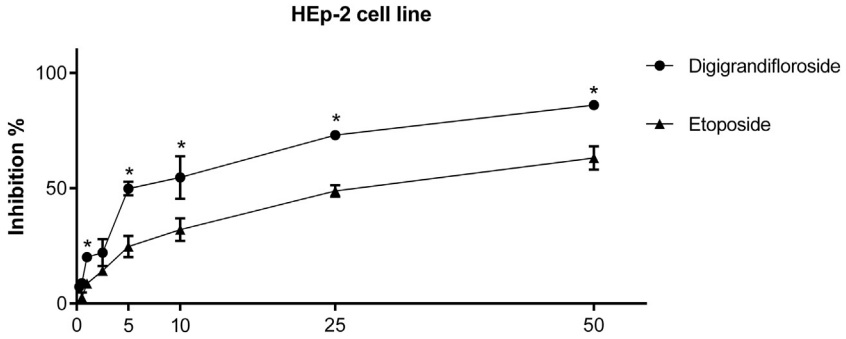

Fig. 4. Screening cytotoxic activity of digigrandifloroside and etoposide on HEp-2 cell line using MTT method. Results are given as inhibition %, and expressed as mean ± S.D. ($n = 3$). *$p < 0.05$ as compared to positive control etoposide.

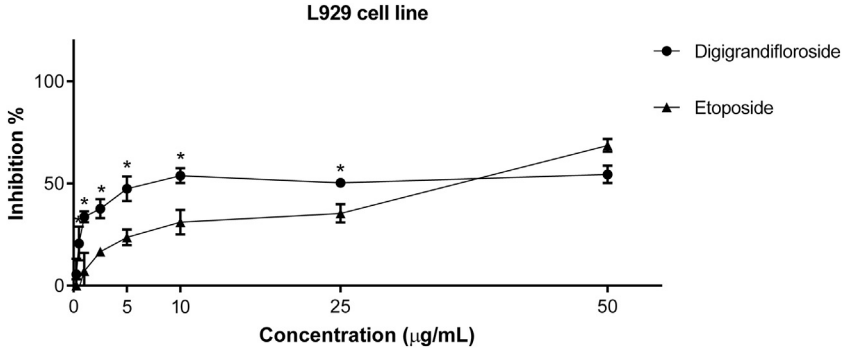

Fig. 5. Screening cytotoxic activity of digigrandifloroside and etoposide on L929 cell line using MTT method. Results are given as inhibition %, and expressed as mean ± S.D. ($n = 3$). *$p < 0.05$ as compared to positive control etoposide.

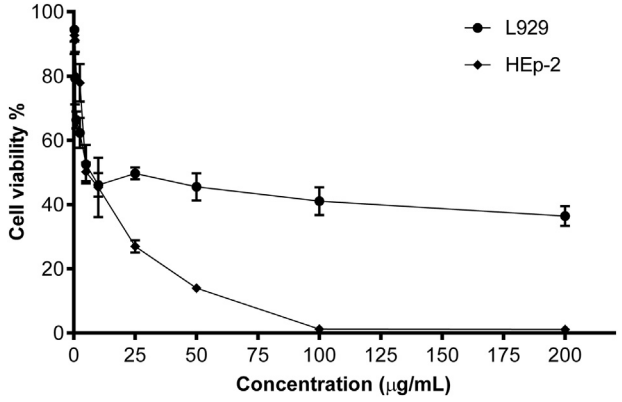

Fig. 6. Comparison of cytotoxic activity of digigrandifloroside on HEp-2 and L929 cell lines. Results are given as cell viability %, and expressed as mean ± S.D. ($n = 3$).

Plantaginaceae family for the first time. In previous studies, rengyoside A was isolated only from Oleaceae (*Forsythia* sp.) and Bignoniacae (*Millingtonia* sp.) families, and rengyoside B was isolated from Oleaceae (*Forsythia* sp.), Bignoniacae (*Incarvillea, Markhamia, Millingtonia, Santisukia, Tecoma* sp.) and Lamiaceae (*Clerodendrum* sp.) families [8,10,12–14,31,34]. This is the second time for cleroindicin A (**4**) being reported from *Digitalis* species, the first report was from *D. trojana* [17]. Previous studies on rengyoside A and B showed that these structures can be afforded by biogenesis like transformation starting from salidroside [6]. Salidroside firstly transform to cornoside, and then rengyosides A and B occur respectively. While salidroside is distributed widely in different plant families, the distribution of rengyosides A and B is restricted. In the future studies, this type of compounds with cleroindicin structure might be studied in detailed with the view of chemotaxonomy of the related families.

Structure of a new cardenolide glycoside named digigrandifloroside (**1**) has been identified by advanced spectral analysis. Cytotoxic activity tests performed on the extract and the isolated compounds. Digigrandifloroside showed strong cytotoxicity on Hep-2 cancer cell line with the IC_{50} value of 10.1 μM when compared with anticancer agent etoposide used as a positive control. Digigrandifloroside also showed selectivity between cancer and noncancerous cell lines. The antiproliferative (cytostatic) effect was seen against noncancerous cells. This selectivity is valuable for the future studies to evaluate the structure activity relationship of the epimerisation in C-12 of the cardenolide glycosides.

Acknowledgement

This work was supported by Hacettepe University Scientific Research Project Coordination Unit (grant number TDK-2016-12687).

Vahap Murat Kutluay was supported by two research grants from The Scientific and Technological Research Council of Turkey (grant numbers TUBITAK 2214/A, 2211).

Authors are thankful for the Kan'ichiro Ishiuchi from Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University (Japan) for operating the NMR and HR-ESIMS analysis and discussion of the digigrandifloroside's structure.

Conflicts of interest

The authors declare that there is no conflict of interest.

References

[1] P. Babula, M. Masarik, V. Adam, I. Provaznik, R. Kizek, From Na+/K+-ATPase and cardiac glycosides to cytotoxicity and cancer treatment, Anticancer Agents Med. Chem. 13 (2013) 1069–1087, https://doi.org/10.2174/18715206113139990304.

[2] C.E. Badr, T. Wurdinger, J. Nilsson, J.M. Niers, M. Whalen, A. Degterev, B.A. Tannous, Lanatoside C sensitizes glioblastoma cells to tumor necrosis factor-

related apoptosisinducing ligand and induces an alternative cell death pathway, Neuro-Oncology 13 (11) (2011) 1213–1224, https://doi.org/10.1093/neuonc/nor067.
[3] M. Benli, N. Yigit, F. Geven, K. Guney, U. Bingol, Antimicrobial activity of endemic *Digitalis lamarckii* Ivan from Turkey, Indian J. Exp. Biol. 47 (2009) 218–221.
[4] J.M. Calderón-Montaño, E. Burgos-Morón, M.L. Orta, D. Maldonado-Navas, I. García-Domínguez, M. López-Lázaro, Evaluating the cancer therapeutic potential of cardiac glycosides, BioMed. Res. Int. 2014 (2014), https://doi.org/10.1155/2014/794930 Article ID 794930.
[5] P.H. Davis, Flora of Turkey and the East Aegean Islands, Vol. 6 University Press, Edinburgh, 1978.
[6] K. Endo, K. Seya, H. Hikino, Biogenesis-like transformation of salidroside to rengyol and its related cyclohexylethanoids of *Forsythia suspensa*, Tetrahedron 45 (1989) 3673–3682, https://doi.org/10.1016/S0040-4020(01)89229-2.
[7] T. Fujino, M. Kuroda, Y. Matsuo, S. Kubo, C. Tamura, N. Sakamoto, Y. Mimaki, M. Hayakawa, Cardenolide glycosides from the seeds of *Digitalis purpurea* exhibit carcinoma-specific cytotoxicity toward renal adenocarcinoma and hepatocellular carcinoma cells, Biosci. Biotechnol. Biochem. 79 (2015) 177–184, https://doi.org/10.1080/09168451.2014.975183.
[8] M. Guiso, C. Marra, F. Piccioni, M. Nicoletti, Iridoid and phenylpropanoid glucosides from *Tecoma capensis*, Phytochemistry 45 (1997) 193–194, https://doi.org/10.1016/S0031-9422(96)00756-X.
[9] G.G. Habermehl, P.E. Hammann, V. Wray, ^{13}C NMR spectra of 5 β,14 β-hydroxysteroids, Magnetic Resonance in Chemistry 23 (1985) 959–963, https://doi.org/10.1002/mrc.1260231115.
[10] T. Hase, Y. Kawamoto, K. Ohtani, R. Kasai, K. Yamasaki, C. Picheansoonthon, Cyclohexylethanoids and related glucosides from *Millingtonia hortensis*, Phytochemistry 39 (1995) 235–241, https://doi.org/10.1016/0031-9422(94)00939-Q.
[11] Q. Jin, H.-G. Jin, J.E. Shin, J. Hong, E.-R. Woo, Phenylethanoid glycosides from *Digitalis purpurea* L, Bull. Korean Chem. Soc. 32 (2011) 1721–1724, https://doi.org/10.5012/bkcs.2011.32. 5.1721.
[12] T. Kanchanapoom, P. Chumsri, R. Kasai, H. Otsuka, K. Yamasaki, A new iridoid diglycoside from *Clerodendrum chinense*, J. Asian Nat. Prod. Res. 7 (2005) 269–272, https://doi.org/10.1080/10286020410001690145.
[13] T. Kanchanapoom, R. Kasai, K. Yamasaki, Phenolic glycosides from *Barnettia kerrii*, Phytochemistry 59 (2002) 565–570, https://doi.org/10.1016/S0031-9422(01)00476-9.
[14] T. Kanchanapoom, R. Kasai, K. Yamasaki, Phenolic glycosides from *Markhamia stipulata*, Phytochemistry 59 (2002) 557–563, https://doi.org/10.1016/S0031-9422(01)00466-6.
[15] A.M. Katz, Effects of *digitalis* on cell biochemistry: sodium pump inhibition, J. Am. Coll. Cardiol. (5) (1985) 16A–21A, https://doi.org/10.1016/S0735-1097(85)80459-9.
[16] H. Kirmizibekmez, Phenylethanoid glycosides from *Digitalis viridiflora*, Rec. Nat. Prod. 9 (2015) 369–373.
[17] H. Kirmizibekmez, M. Masullo, M. Festa, A. Capasso, S. Piacente, Steroidal glycosides with antiproliferative activities from *Digitalis trojana*, Phytother. Res. 28 (2014) 534–538, https://doi.org/10.1002/ptr.5012.
[18] W. Kreis, The Foxgloves (*Digitalis*) revisited, Planta Med. 83 (2017) 962–976, https://doi.org/10.1055/s-0043-111240.
[19] H. Kuwajima, Y. Takai, K. Takaishi, K. Inoue, Synthesis of ^{13}C-labeled possible intermediates in the biosynthesis of phenylethanoid derivatives, cornoside and rengyosides, Chem. Pharm. Bull. 46 (1998) 581–586, https://doi.org/10.1248/cpb.46.581.
[20] X. Li, Y. Ren, Y. Bao, J. Liu, X. Zhang, Y. Zhang, X. Sun, X. Yao, J. Tang, Synthesis of C_3-Neoglycosides of digoxigenin and their anticancer activities, Eur J Med Chem 145 (2018) 252–262, https://doi.org/10.1016/j.ejmech.2017.12.086.
[21] M. Lopez-Lazaro, D.L.P.N. Palma, N. Pastor, C. Martin-Cordero, E. Navarro, F. Cortes, M.J. Ayuso, M.V. Toro, Anti-tumour activity of *Digitalis purpurea* L. subsp. *heywoodii*, Planta Med 69 (2003) 701–704, https://doi.org/10.1055/s-2003-42789.
[22] T. Mijatovic, E. Van Quaquebeke, B. Delest, O. Debeir, F. Darro, R. Kiss, Cardiotonic steroids on the road to anti-cancer therapy, Bba-Rev Cancer 1776 (2007) 32–57, https://doi.org/10.1016/j.bbcan.2007.06.002.
[23] D.J. Newman, G.M. Cragg, Natural products as sources of new drugs from 1981 to 2014, J Nat Prod 79 (2016) 629–661, https://doi.org/10.1021/acs.jnatprod.5b01055.
[24] J.W. Oh, J.Y. Lee, S.H. Han, Y.H. Moon, Y.G. Kim, E.-R. Woo, K.W. Kang, Effects of phenylethanoid glycosides from *Digitalis purpurea* L. on the expression of inducible nitric oxide synthase, J. Pharm. Pharmacol. 57 (2005) 903–910, https://doi.org/10.1211/0022357056451.
[25] I. Orhan, D. Deliorman-Orhan, B. Özçelik, Antiviral activity and cytotoxicity of the lipophilic extracts of various edible plants and their fatty acids, Food Chem. 115 (2009) 701–705, https://doi.org/10.1016/j.foodchem.2009.01.024.
[26] R.M. Padua, A.B. Oliveira, J.D.S. Filho, G.J. Vieira, J.A. Takahashi, F.C. Braga, Biotransformation of digitoxigenin by *Fusarium ciliatum*, J. Braz. Chem. Soc. 16 (2005) 614–619, https://doi.org/10.1590/S0103-50532005000400019.
[27] A. Perrone, A. Capasso, M. Festa, E. Kemertelidze, C. Pizza, A. Skhirtladze, S. Piacente, Antiproliferative steroidal glycosides from *Digitalis ciliata*, Fitoterapia 83 (2012) 554–562, https://doi.org/10.1016/j.fitote.2011.12.020.
[28] M. Ramirez-Ortega, V. Maldonado-Lagunas, J. Melendez-Zajgla, J.F. Carrillo-Hernandez, G. Pastelin-Hernandez, O. Picazo-Picazo, G. Ceballos-Reyes, Proliferation and apoptosis of HeLa cells induced by *in vitro* stimulation with digitalis, Eur. J. Pharmacol. 534 (2006) 71–76, https://doi.org/10.1016/j.ejphar.2006.01.035.
[29] I. Saracoglu, M. Inoue, I. Calis, Y. Ogihara, Studies on constituents with cytotoxic and cytostatic activity of two Turkish medicinal plants *Phlomis armeniaca* and *Scutellaria salviifolia*, Biol. Pharm. Bull. 18 (1995) 1396–1400, https://doi.org/10.1248/bpb.18.1396.
[30] N.F.Z. Schneider, C. Cerella, J.Y. Lee, A. Mazumder, K.R. Kim, A. de Carvalho, J. Munkert, R.M. Padua, W. Kreis, K.W. Kim, C. Christov, M. Dicato, H.J. Kim, B.W. Han, F.C. Braga, C.M.O. Simoes, M. Diederich, Cardiac glycoside glucoevatromonoside induces cancer type-specific cell death, Front Pharmacol 9 (2018), https://doi.org/10.3389/fphar.2018.00070.
[31] K. Seya, K. Endo, H. Hikino, Structures of rengyosides A, B, and C, three glucosides of *Forsythia suspensa* fruits, Phytochemistry 28 (1989) 1495–1498, https://doi.org/10.1016/S0031-9422(00)97772-0.
[32] J. Tian, Q.-S. Zhao, H.-J. Zhang, Z.-W. Lin, H.-D. Sun, New cleroindicins from *Clerodendrum indicum*, J. Nat. Prod. 60 (1997) 766–769, https://doi.org/10.1021/np9606759.
[33] S.J. Warr, K. Thompson, M. Kent, Antifungal activity in seed coat extracts of woodland plants, Oecologia 92 (1992) 296–298, https://doi.org/10.1007/BF00317378.
[34] J. Wu, X. Zhou, X. Zhou, S. Huang, C. Wang, Chemical constituents from flowers of Incarvillea younghusbandii, Zhongcaoyao 43 (2012) 55–59.