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Antiproliferative steroidal glycosides from *Digitalis ciliata*

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ABSTRACT

Two new compounds, a furostanol glycoside (**1**) and a pregnane glycoside (**4**), along with eight known compounds, belonging to the classes of spirostane (**2**,**3**), pregnane (**5**–**7**) and cardenolide (**8**–**10**) glycosides, were isolated from the seeds of *Digitalis ciliata*. Their structures were elucidated by 1D and 2D-NMR experiments as well as ESI-MS analysis. For the first time pregnane glycosides of the diginigenin series have been isolated from *D. ciliata*. The cytotoxic effects of compounds **1**–**10** on cell viability of several cancer cell lines, namely human breast cancer (MCF-7), human glioblastoma (T98G), human lung adenocarcinoma (A549), human colon carcinoma (HT-29), and human prostate cancer (PC-3) cell lines were evaluated. Compounds **1**, **4**, **7** and **8** showed antiproliferative effects against MCF-7, HT-29 and A549 cancer cells with IC_{50} values ranging from 8.3 to 20 μM. The effects of compounds **1**–**10** on cell proliferation were evaluated on these three cancer cell lines by cell cycle analysis of DNA content using flow cytometry. Compounds **7**, **8** and **10** induced significant changes in G_2/M cell cycle phase of all analyzed cells. The obtained results indicate that compounds **7**, **8** and **10** are cytostatic compounds effective in reducing cell proliferation by inducing accumulation of the cells in the G_2/M phase of the cell cycle.

1. Introduction

Cardiac glycosides are one of the most important classes of biologically active natural compounds; the leading place belongs to foxglove (*Digitalis*) cardenolides, which have been successfully used in medicine for more than two centuries. The genus *Digitalis* comprises 36 species, common to many countries, among them *Digitalis ciliata* Trautv. (Scrophulariaceae), known also as ciliate foxglove, is a perennial herb which is widely distributed in alpine meadows of Georgia and is strictly endemic to both the Republic and the whole Caucases region [1]. *D. ciliata* is rich in cardenolide glycosides and biosynthesizes glycosides of almost all genins typical of this genus: digitoxigenin, gitoxigenin, digoxigenin, gitaloxigenin, and diginatigenin [2]. It appeared to be a high-grade medical raw material due to the presence of about 50 cardenolides with total product yield up to 4–4.5% [2].

D. ciliata has been proposed as a raw material to produce an effective cardiotonic preparation of acetyldigitoxin [3].

Steroidal glycosides of the 5α-spirostane and 5α-furostane classes, derivatives of gitogenin and tigogenin, have been isolated from aqueous wastes from production of the drug acetyldigitoxin from the leaves of ciliate foxglove [1–3].

There are several reports on the cytotoxic activity of cardiac glycosides, and a potential anticancer effect has been suggested. Cytotoxic effects of these glycosides against a number of types of cancer cell lines have also been demonstrated in vitro. Several reports indicate that malignant cells are more susceptible to the effects of cardiac glycosides compared to normal cells [4,5].

Compounds belonging to the class of furostane- and spirostane-type steroidal saponins have displayed cytotoxic activity [6–8]. A recent research reports that saponins have antitumor effect on many cancer cells: several saponins inhibit tumor cell growth by cell cycle arrest and apoptosis [9].

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The interesting biological activity showed by these two classes of metabolites and the occurrence these compounds in *D. ciliata*, prompted us to carry out their isolation from this species and to evaluate their cytotoxic activity. Therefore, we investigated the seed extract of *D. ciliata*, and here we report a furostanol glycoside (**1**) and a pregnane glycoside (**4**), along with eight known compounds (**2**, **3**, **5**–**10**) (Fig. 1).

Their structures were elucidated by spectroscopic methods including 1D-(^{1}H, 1D-TOCSY, ^{13}C) and 2D-NMR (DQF-COSY, HSQC, HMBC, ROESY) experiments as well as ESI-MS and HRMALDITOFMS analysis.

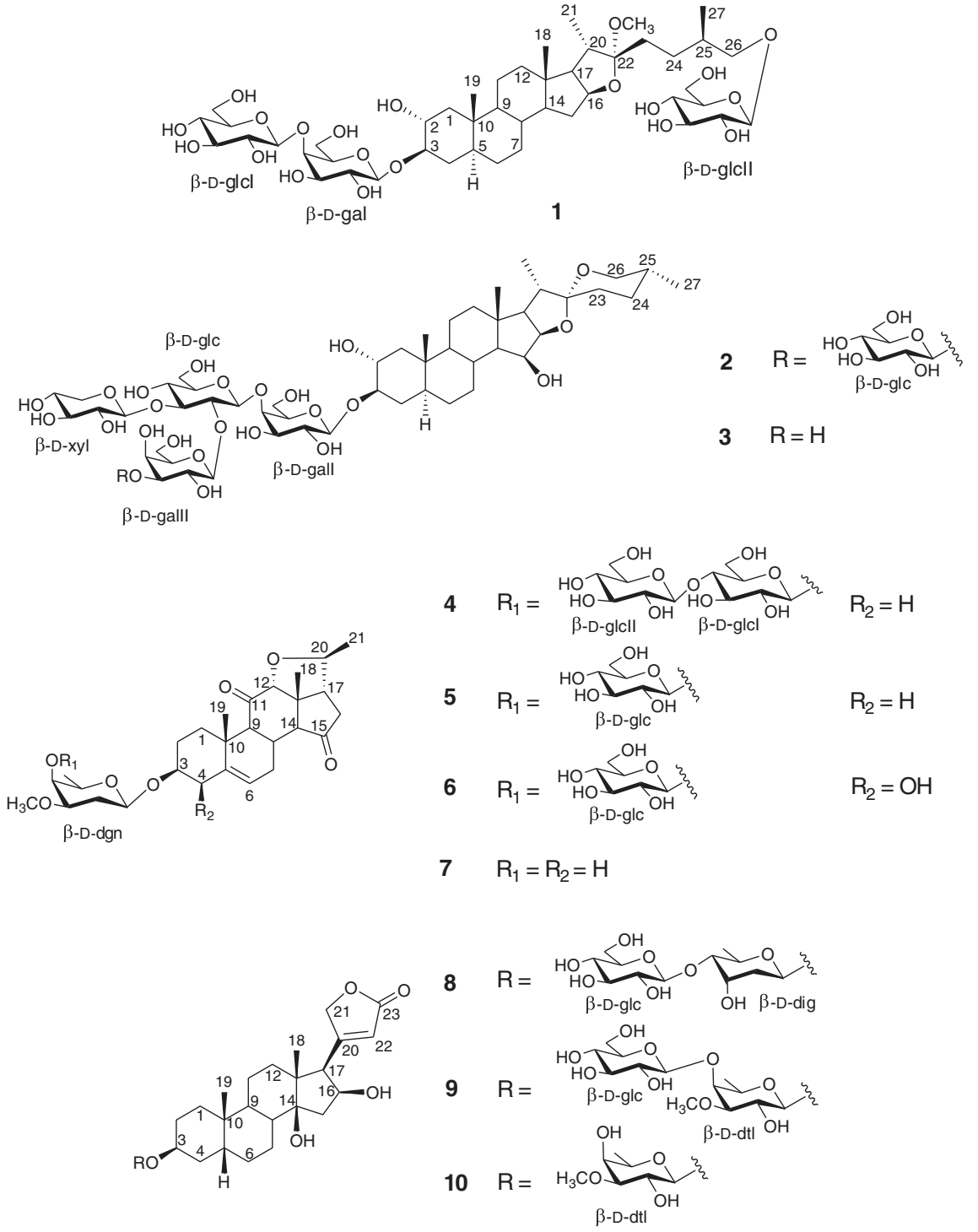

Fig. 1. Secondary metabolites (**1**–**10**) isolated from of *D. ciliata*.

The cytotoxic effects of compounds **1**–**10** on cell viability of several cancer cell lines, human breast cancer (MCF-7), human glioblastoma (T98G), human lung adenocarcinoma (A549), human colon carcinoma (HT-29), and human prostate cancer (PC-3) cell lines were evaluated.

2. Experimental

2.1. General

Optical rotations were measured on a JASCO DIP 1000 polarimeter. IR measurements were obtained on a Bruker IFS-48 spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpinGmBH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe at 300 K. All 2D-NMR spectra were acquired in CD_3OD (99.95%, Sigma Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC and ROESY spectra. The NMR data were processed using UXNMR software. Exact masses were measured by a Voyager DE mass spectrometer. Samples were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDITOF) mass spectrometry. A mixture of analyte solution and a-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18–39) at 2465.1989 Da and α-cyano-4-hydroxycinnamic acid at 190.0504 as internal standards.

Column chromatography was performed over Silica gel (100×2.5 cm, 60 μm, Merck). TLC was performed on silica gel plates (Merck precoated silica gel 60 F_{254}) and developed in the solvent system, $CHCl_3$:MeOH:H_2O (26:14:3). The spots were visualized by UV radiation and cerium sulfate solution spraying, followed by heating at 150 °C.

HPLC separations were carried out on a Waters 590 system equipped with a Waters R401 refractive index detector, a Waters XTerra Prep MSC_{18} column (300×7.8 mm i.d.) and a Rheodyne injector. All solvents for extraction and chromatographic separation were of analytical grade and purchased from Carlo Erba (Rodano, Italy). HPLC grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA).

2.2. Plant material

The seeds of *D. ciliata* were collected in September 2008 in north-west Georgia (Svaneti region). Samples of *D. ciliata* were identified by Dr. Jemal Aneli, Department of Pharmacobotany, Institute of Pharmacochemistry, Tbilisi, Georgia. A voucher specimen (No. 118) has been deposited at this department.

2.3. Extraction and isolation

150 g powdered seeds of *D. ciliata* were defatted with petroleum ether and then extracted with chloroform in Soxhlet to yield 46 g oil and 12 g extract, respectively. The seeds were dried and extracted with MeOH 80% three times. The collected extracts have been dried under vacuum and the concentrate was partitioned between *n*-BuOH (10.5 g) and water (5.4 g). Part of chloroform extract (3 g) was subjected to a silica gel column chromatography (100×2.5 cm, 60 μm, Merck) eluting with gradient system chloroform–methanol-(10:0→0:10). Fractions containing steroidal glycosides were selected for further purification and were combined in fractions A (64 mg), B (109 mg), C (211 mg), and D (17 mg). Fractions A–D were chromatographed by RP-HPLC (Waters XTerra Prep MSC18 column, 300×7.8 mm i.d.), at flow rate 2.0 ml/min, using different mixtures of MeOH: H_2O in isocratic conditions. From fraction A using 47% MeOH as mobile phase compound **5** (4.7 mg, t_R = 14.6 min) was obtained; from fraction B using 42% MeOH as mobile phase compounds **9** (0.6 mg, t_R = 18.8 min) and **7** (0.9 mg, t_R = 32.5 min) were isolated; while from fraction C using 42% MeOH mobile phase yielded compounds **4** (2.2 mg, t_R = 17.5 min), **6** (5.7 mg, t_R = 19.2 min), **8** (1.4 mg, t_R = 34.6 min) and from fraction D using 38% MeOH as mobile phase compound **10** (0.2 mg, t_R = 26.9 min). Part of *n*-buthanolic extract (2 g) was subjected to a silica gel column chromatography (100×2.5 cm, 60 μm, Merck) eluting with isocratic system chloroform–methanol–water 26:14:3 yielding 52 fractions (8 mL each) which were combined. The major fraction 19–23 (151 mg) was chromatographed by RP-HPLC (Waters XTerra Prep MSC18 column, 300×7.8 mm i.d.), at flow rate 2.0 mL/min, using 57% MeOH as mobile phase, and compounds **1** (1.2 mg, t_R = 14.2 min), **2** (3.7 mg, t_R = 46.5 min) and **3** (3.8 mg, t_R = 54.2 min) were obtained.

2.3.1. (25R)-26-O-β-D-glucopyranosyl-5α-furostan-2α,3β,22α,26-tetraol 3-O-[β-D-glucopyranosyl-(1→4)-O-β-D-galactopyranoside] (**1**)

Amorphous white powder; $[\alpha]^{22}_D$ + 10.6° (*c* 0.1 MeOH); IR (KBr): ν_{max} = 3429, 2927, 1265 and 1048 cm^{-1}; HRMALDI-TOFMS *m/z* $[M+Na]^+$ calcd for $C_{45}H_{72}O_{19}Na$, 973.4984, found 973.4989; ESI-MS *m/z* = 973.5 $[M+Na]^+$, ESI-MS/MS *m/z* = 941.5 $[M+Na\text{-}32]^+$, 811.5 $[M+Na\text{-}162]^+$; ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of aglycon moiety: Table 1; ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of the sugar portion: Table 2.

2.3.2. (25R)-5α-spirostan-2α,3β,15β-triol 3-O-β-D-galactopyranosyl-(1→2)-O-[β-D-xylopyranosyl-(1→3)]-O-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (desglucodigitonin) (**3**)

Amorphous white powder; $[\alpha]^{22}_D$ − 40.8° (*c* 0.1 MeOH); IR (KBr): ν_{max} = 3438, 2932, 1250 and 1065 cm^{-1}; HRMALDITOFMS *m/z* $[M+Na]^+$ calcd for $C_{50}H_{82}O_{24}Na$, 1089.5094, found 1089.5099; ESI-MS *m/z* = 1089.6 $[M+Na]^+$, ESI-MS/MS *m/z* = 927.4 $[M+Na\text{-}162]^+$, 795.4 $[M+Na\text{-}162\text{-}132]^+$, 633.4 $[M+Na\text{-}162\text{-}132\text{-}162]^+$; ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of aglycon moiety: Table 1; ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of the sugar portion: Table 2.

2.3.3. 12α,20α-epoxy-3β-hydroxy-14β,17α-pregn-5-en-11,15-dione 3-O-[β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-O-β-D-diginopyranoside] (**4**)

Amorphous white powder; $[\alpha]^{22}_D$ − 184° (*c* 0.1 MeOH); IR (KBr): ν_{max} 3450, 2938, 1754, 1259 and 1040 cm^{-1}; HRMALDITOFMS *m/z* $[M+Na]^+$ calcd for $C_{40}H_{60}O_{17}Na$, 835.3728, found 835.3732; ESI-MS *m/z* = 835.6 $[M+Na]^+$, ESI-MS/MS *m/z* = 673.3 $[M+Na\text{-}162]^+$, 511.3 $[M+Na\text{-}162\text{-}162]^+$, 367.1 $[M+Na\text{-}162\text{-}162\text{-}144]^+$; ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of aglycon moiety: Table 1; ^1H and ^{13}C NMR (CD_3OD, 600 MHz) data of the sugar portion: Table 2.

Table 1
^{13}C and ^{1}H NMR data (*J* in Hz) of the aglycon moiety of compounds **1**, **3** and **4** (600 MHz, CD_3OD).[a].

Position	1		3		4	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	45.9	2.02, 0.96 *m*	45.6	2.01, 0.97 *m*	38.4	2.09, 1.30 *m*
2	71.1	3.66 *m*	71.3	3.70 *m*	30.0	1.96, 1.58 *m*
3	84.8	3.49 *m*	84.5	3.52 *m*	79.0	3.58 *m*
4	33.7	1.77, 1.43 *m*	33.6	1.78, 1.45 *m*	39.2	2.46, 2.18 *m*
5	45.6	1.21 *m*	46.0	1.24 *m*	141.4	–
6	29.2	1.40 (2H) *m*	29.0	1.42, 1.37 *m*	123.3	5.53 *br d* (5.5)
7	33.4	1.77, 0.98 *m*	31.4	1.74 (2H) *m*	30.3	2.73, 2.10 *m*
8	35.8	1.59 *m*	43.1	1.95 *m*	32.0	2.54 *m*
9	55.4	0.78 *m*	55.8	0.84 *m*	59.7	1.92 *d* (13.3)
10	38.2	–	37.8	–	40.3	–
11	22.0	1.59, 1.41 *m*	22.5	1.59, 1.48 *m*	215.1	–
12	40.8	1.78, 1.18 *m*	43.4	1.71, 1.25 *m*	90.0	4.02 *s*
13	41.4	–	41.4	–	40.0	–
14	57.2	1.16 *m*	61.1	1.10 *dd* (11.3, 3.8)	57.8	2.58 *d* (4.8)
15	32.5	2.00, 1.19 *m*	70.8	4.15 *dd* (5.6, 3.8)	220.1	–
16	82.2	4.39 *dd* (14.5, 7.5)	83.7	4.33 *dd* (8.7, 5.6)	40.1	2.28 *dd* (19.1, 8.8) 1.81 *dd* (19.1, 9.9)
17	64.9	1.76 *m*	62.8	1.95 *d* (8.7)	49.5	2.51 *ddd* (9.9, 8.8, 4.9)
18	16.8	0.85 *s*	18.9	1.06 *s*	25.3	1.61 *s*
19	13.4	0.92 *s*	13.7	0.95 *s*	19.8	1.07 *s*
20	40.9	2.20 *m*	43.3	1.99 *dd* (8.7, 6.6)	77.6	4.63 *dd* (6.5, 4.9)
21	15.6	1.03 *d* (6.6)	14.7	0.98 *d* (6.6)	17.1	1.27 *d* (6.5)
22	113.9	–	110.8	–		
23	31.1	1.86, 1.64 *m*	32.5	2.01, 1.05 *m*		
24	29.0	1.62, 1.18 *m*	29.5	1.68, 1.54 *m*		
25	35.1	1.77 *m*	31.4	1.65 *m*		
26	75.7	3.76, 3.42 *m*	67.8	3.51, 3.41 *m*		
27	17.0	0.98 *d* (6.6)	17.4	0.83 *d* (6.3)		
OMe	47.6	3.17 *s*				

[a] Assignments were confirmed by 1D-TOCSY, DQF-COSY, HSQC and HMBC experiments.

2.4. Acid hydrolysis

A solution (0.8 mg each) of **1**, **3** and **4** was refluxed with 0.25 mL of 2 N HCl for 4 h. The aglycons were extracted with EtOAc (3×15 mL), and the organic layer was neutralized by washing with H_2O, and evaporated to dryness. The acid aqueous layer was neutralized with 1 N NaOH and freeze-dried. Sugars were identified with authentic samples by TLC in MeCOEt–isoPrOH–Me_2CO–H_2O (20:10:7:6) as diginose, xylose, glucose and galactose. After a preparative TLC of sugar mixture (50 mg) in this solvent, the optical rotation of each purified sugar was measured. The D configuration of galactose and glucose was established by comparison of their optical rotation values with those reported in the literature: D-glucose $[\alpha]^{23}_D + 52.5$, D-galactose $[\alpha]^{23}_D + 80.2$, D-xylose $[\alpha]^{23}_D + 18.8$ [10], D-diginose $[\alpha]^{23}_D + 59.6$ [11]. The optical rotations were determined after dissolving the sugars in H_2O and allowing them to stand for 24 h: D-glucose $[\alpha]^{23}_D + 53.2$ (*c* 0.1), D-galactose $[\alpha]^{23}_D + 81.1$ (*c* 0.1), D-diginose $[\alpha]^{23}_D + 60.5$ (*c* 0.1), D-xylose $[\alpha]^{23}_D + 19.2$.

2.5. Cell cultures

Human breast (MCF-7), glioblastoma (T98G), lung (A549), colon (HT-29), and prostate (PC-3) cancer cells were cultured in DMEM medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (all from Cambrex Bioscience, Verviers, Belgium) at 37 °C in an atmosphere of 95% O_2 and 5% CO_2. The cells were used up to a maximum of 10 passages.

2.6. MTT bioassay

Human cancer cells (3×10^3) were plated in 96-well culture plates in 90 μL of culture medium and incubated at 37 °C in humidified 5% CO_2. The day after, 10 μL aliquot of serial dilutions of compounds **1**–**10** (1–50 μM), staurosporine used as positive control (1–5 μM) and quercetin used as negative control (1–50) μM were added to the cells and incubated for 48 h. Cell viability was assessed through MTT assay [12]. Briefly, 25 μL of MTT (5 mg/mL) were added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 μL of a solution containing 50% (v:v) N, N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. Cell viability in response to treatment was calculated as percentage of control cells treated with solvent DMSO at the final concentration 0.1%: % viable cells = (100×OD treated cells)/OD control cells.

Staurosporine and quercetin were purchased from Sigma-Aldrich.

2.7. Analysis of apoptosis and necrosis

Hypodiploid DNA was analyzed using the method of propidium iodide (PI) staining and flow cytometry as described

Table 2
^{13}C and ^{1}H NMR data (*J* in Hz) of the sugar portions of compounds **1**, **3** and **4** (600 MHz, CD_3OD).[a].

Position	1		3		4	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
	β-D-Gal		β-D-Gall		β-D-Dgn	
1	102.8	4.38 *d* (7.9)	102.2	4.41 *d* (7.9)	99.5	4.68 *dd* (9.8, 1.8)
2	72.7	3.59 *dd* (8.5, 7.9)	72.3	3.84 *dd* (8.5, 7.9)	33.4	1.98 *ddd* (13.0, 4.4, 1.8)
						1.80 *ddd* (13.0, 12.1, 9.8)
3	75.0	3.62 *dd* (8.5, 2.9)	75.3	3.57 *dd* (8.5, 2.9)	80.4	3.51 *ddd* (2.4, 4.4, 12.1)
4	79.2	4.08 *dd* (2.9, 1.2)	79.9	4.07 *dd* (2.9, 1.2)	74.0	4.03 *br s*
5	75.6	3.59 *m*	75.3	3.59 *m*	71.4	3.56 *m*
6	60.9	3.87 *dd* (12.0, 2.0)	61.0	3.89 *dd* (12.0, 2.0)	17.5	1.32 *d* (6.3)
		3.70 *dd* (12.0, 4.5)		3.69 *dd* (12.0, 4.5)		
-OMe					56.2	3.43 *s*
	β-D-GlcI		β-D-GlcI		β-D-GlcI	
1	106.1	4.55 *d* (7.9)	104.2	4.65 *d* (7.9)	103.7	4.66 *d* (7.9)
2	75.4	3.30 *dd* (9.0, 7.9)	80.6	3.80 *dd* (9.0, 7.9)	75.4	3.31 *dd* (9.0, 7.9)
3	77.8	3.36 *dd* (9.0, 9.0)	87.2	3.75 *dd* (9.0, 9.0)	75.8	3.56 *dd* (9.0, 9.0)
4	71.7	3.25 *dd* (9.0, 9.0)	70.0	3.31 *dd* (9.0, 9.0)	80.6	3.59 *dd* (9.0, 9.0)
5	77.8	3.31 *m*	78.0	3.33 *m*	76.5	3.41 *m*
6	62.9	3.93 *dd* (12.0, 2.5)	62.8	3.93 *dd* (12.0, 2.5)	62.0	3.88 *dd* (12.0, 2.5)
		3.63 *dd* (12.0, 4.5)		3.61 *dd* (12.0, 4.5)		3.69 *dd* (12.0, 4.5)
	β-D-GlcII		β-D-GallI		β-D-GlcII	
1	104.2	4.27 *d* (7.9)	104.5	4.92 *d* (7.9)	104.2	4.44 *d* (7.9)
2	75.0	3.22 *dd* (9.0, 7.9)	72.9	3.59 *dd* (9.8, 7.9)	74.7	3.25 *dd* (9.0, 7.9)
3	77.9	3.39 *dd* (9.0, 9.0)	74.5	3.51 *dd* (9.8, 3.4)	77.6	3.39 *dd* (9.0, 9.0)
4	71.4	3.31 *dd* (9.0, 9.0)	70.2	3.88 *dd* (3.4, 1.1)	71.1	3.35 *dd* (9.0, 9.0)
5	77.6	3.28 *m*	77.0	3.61 *m*	77.8	3.36 *m*
6	62.6	3.90 *dd* (12.0, 2.5)	62.4	4.04 *dd* (11.8, 7.0)	62.0	3.90 *dd* (12.0, 2.5)
		3.70 *dd* (12.0, 4.5)		3.74 *dd* (11.8, 5.9)		3.69 *dd* (12.0, 4.5)
			β-D-Xyl			
1			104.2	4.64 *d* (7.5)		
2			75.0	3.27 *dd* (9.0, 7.5)		
3			77.5	3.37 *dd* (9.0, 9.0)		
4			70.8	3.55 *m*		
5			67.0	3.95 *dd* (10.5, 4.5)		
				3.29 *t* (10.5)		

[a] Assignments were confirmed by 1D-TOCSY, DQF-COSY, HSQC and HMBC experiments.

[13]. Briefly, cells were washed in phosphate-buffered saline (PBS) and resuspended in 500 μL of a solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/mL propidium iodide (Sigma-Aldrich, Italy). After incubation at 4 °C for 30 min in the dark, cell nuclei were analyzed with Becton Dickinson FACScan flow cytometer using the Cells Quest program. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on logarithmic scale. The percentage of the cells in the hypodiploid region was calculated. Necrosis was quantified by PI staining of non-permeabilized cells. Cells were collected, washed twice with PBS and then resuspended in 4 mg/mL PI/PBS solution and then immediately analyzed by flow cytometry. Data from 10,000 events per sample were collected and analyzed using CellQuest software.

2.8. Cell cycle analysis

Cells were plated at 1×10^5 in 60 mm dish and exposed to increasing concentrations of compounds **6**, **7**, **8** and **10**. After the incubation period cells were harvested and fixed in cold 70% ethanol at −20 °C. Cell cycle profiles were evaluated by DNA staining with 2.5 mg/mL propidium iodide in phosphate buffered saline (PBS) supplemented with 100 U/mL ribonucleases A, for 30 min at room temperature. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson, CA) using the Cells Quest evaluation program. Distribution of cells in distinct cell cycle phases was determinated using ModFit LT cell cycle analysis software.

2.9. Statistical analysis

All results are shown as mean ± SEM of three experiments performed in triplicate. Statistical comparison between groups was made using ANOVA followed by Bonferroni parametric test. Differences were considered significant if $P<0.05$.

3. Results and discussion

After defatting, the seeds of *D. ciliata* were extracted with $CHCl_3$ and 80% MeOH and each extract was fractionated over silica gel column. The obtained fractions were chromatographed by reversed-phase HPLC to yield two new compounds, **1** and **4**, along with the known compounds **2**, **3** and **5**–**10** (see Experimental section).

The molecular formula of **1** was unequivocally established to be $C_{46}H_{78}O_{20}$ by HRMALDITOFMS (*m/z* 973.4989 $[M+Na]^+$, calcd for $C_{45}H_{72}O_{19}Na$, 973.4984). The positive ESIMS mass spectrum of **1** showed the major ion peak at *m/z* 973.5 which was assigned to $[M+Na]^+$. The MS/MS of this ion showed peaks at *m/z* 941.5 $[M+Na\text{-}32]^+$, due to the loss of a methoxy

group, and at *m/z* 811.5 $[M+Na\text{-}162]^+$ corresponding to the loss of a hexose unit.

The ^1H NMR spectrum of **1** showed signals for two tertiary methyl groups at δ 0.85 (3H, *s*) and 0.92 (3H, *s*), two secondary methyl groups at δ 0.98 (3H, *d*, *J* = 6.6 Hz) and 1.03 (3H, *d*, *J* = 6.6 Hz), three methine proton signals at δ 3.49 (1H, *m*), 3.66 (1H, *m*) and 4.39 (1H, *dd*, *J* = 14.5, 7.5) indicative of secondary alcoholic functions, two methylene proton signals at δ 3.42 and 3.76 (each 1H, *m*) ascribable to a primary alcoholic function, and three anomeric protons at δ 4.55 (1H, *d*, *J* = 7.9 Hz), 4.38 (1H, *d*, *J* = 7.9 Hz) and 4.27 (1H, *d*, *J* = 7.9 Hz). The ^{13}C NMR spectrum displayed signals ascribable to a hemiacetal function at δ 113.9, three secondary alcoholic functions at δ 71.1, 82.2 and 84.8, and one primary alcoholic function at δ 75.7, suggesting the occurrence of a glycosidic furostanol skeleton (see Table 1). On the basis of the HSQC and HMBC correlations, the aglycon moiety of compound **1** was identified as (25*R*)-5α-furostan-2α,3β,22α,26-tetraol. The configuration of the hydroxyl group at C-22 was established to be α from ROESY correlations between H-20 (δ 2.20) and the protons H-23a (δ 1.86) and H-23b (δ 1.64). The C-25 configuration was deduced to be *R* based on the difference of chemical shifts ($\Delta_{ab} = \delta_a - \delta_b$) of the geminal protons at H_2-26 (Δ_{ab} = 0.34 ppm). It has been described that Δ_{ab} is usually >0.57 ppm in 25*S* compounds and <0.48 in 25*R* compounds [14]. It was evident from the ^1H and ^{13}C NMR data that the sugar chain of **1** consisted of three sugar units. The chemical shifts of all the individual protons of the three sugar units were ascertained from a combination of 1D-TOCSY and DQF-COSY spectral analysis, and the ^{13}C chemical shifts of their relative attached carbons were assigned unambiguously from the HSQC spectrum (see Table 2). These data showed the presence of two β-glucopyranosyl units (δ 4.55 and 4.27) and one β-galactopyranosyl unit (δ 4.38). Glycosidation shift was observed for C-4_{gal} (δ 79.2). An unambiguous determination of the sequence and linkage sites was obtained from the HMBC spectrum, which showed key correlation peaks between the proton signal at δ 4.38 (H-1_{gal}) and the carbon resonance at δ 84.8 (C-3), the proton signal at 4.55 (H-1_{glcI}) and the carbon resonance at 79.2 (C-4_{gal}) and the proton signal at δ 4.7 (H-1_{glcII}) and the carbon resonance at δ 75.7 (C-26).

On the basis of all this evidence, the structure of the new compound **1** was established as (25*R*)-26-*O*-β-D-Xglucopyranosyl-5α-furostan-2α,3β,22α,26-tetraol 3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside].

Compound **3** showed in the positive ESIMS a major ion peak at *m/z* 1089.6 $[M+Na]^+$ and significant fragments in MS/MS analysis at *m/z* 927.4 $[M+Na\text{-}162]^+$, ascribable to the loss of a hexose unit, at *m/z* 795.4 $[M+Na\text{-}162\text{-}132]^+$ due to the loss of a pentose unit and at *m/z* 633.4 $[M+Na\text{-}162\text{-}132\text{-}162]^+$, indicating the loss of a second hexose unit. Its molecular formula was established unequivocally as $C_{50}H_{82}O_{24}$ by HRMALDITOF mass spectrum (*m/z* 1089.5099 $[M+Na]^+$, calcd. for $C_{50}H_{82}O_{24}Na$, 1089.5094).

The ^1H NMR spectrum of **3** showed signals for two tertiary methyl groups at δ 0.95 (3H, *s*) and 1.06 (3H, *s*), two secondary methyl groups at δ 0.83 (3H, *d*, *J* = 6.3 Hz) and 0.98 (3H, *d*, *J* = 6.6 Hz), four methine proton signals at δ 3.52 (1H, *m*), 3.70 (1H, *m*), 4.15 (1H. *dd*, *J* = 5.6, 3.8 Hz) and 4.33 (1H. *dd*, *J* = 8.7, 5.6 Hz) indicative of secondary alcoholic functions, two methylene proton signals at δ 3.51 and 3.41 (each 1H, *m*) ascribable to a primary alcoholic function, and four anomeric protons at δ 4.92 (1H, *d*, *J* = 7.9 Hz), 4.65 (1H, *d*, *J* = 7.9 Hz), 4.64 (1H, *d*, *J* = 7.5 Hz) and 4.41 (1H, *d*, *J* = 7.9 Hz). A detailed analysis of NMR data of compound **3** in comparison with those reported for digitonin (**2**), previously isolated from *Digitalis* spp. [15], showed that compound **3** differed from digitonin only by the absence of the glucose unit at C-3 of the second galactose unit in the sugar chain. Thus, compound **3** was (25*R*)-5α-spirostan-2α,3β,15β-triol 3-*O*-β-D-galactopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside, namely desglucodigitonin (**3**), previously isolated only from the commercial "digitonin", a complex mixture of structurally related saponins [16].

The HRMALDITOF mass spectrum of compound **4** (*m/z* 835.3732 $[M+Na]^+$, calcd. for $C_{40}H_{60}O_{17}Na$, 835.3728) supported a molecular formula of $C_{40}H_{60}O_{17}$. The ESIMS mass spectrum showed the major ion peak at *m/z* 835.6 which was assigned to $[M+Na]^+$. In the MS/MS spectrum peaks at *m/z* 673.3 $[M+Na\text{-}162]^+$, corresponding to the loss of a hexose unit, at *m/z* 511.3 $[M+Na\text{-}162\text{-}162]^+$, corresponding to the loss of a second hexose unit, and at *m/z* 367.1 $[M+Na\text{-}162\text{-}162\text{-}144]^+$, due to the loss of a methyldideoxyhexose unit, were observed.

The ^1H NMR spectrum of **4** showed signals for two tertiary methyl groups at δ 1.07 (3H, *s*) and 1.61 (3H, *s*), one secondary methyl group at δ 1.27 (3H, *d*, *J* = 6.5 Hz), three methine proton signals at δ 3.58 (1H, *m*), 4.02 (1H, *s*) and 4.63 (1H, *dd*, *J* = 6.5, 4.9 Hz) indicative of secondary alcoholic function. In addition, the ^{13}C NMR spectrum showed for the aglycon moiety 21 signals suggesting the presence of a pregnane skeleton [17]. The ^{13}C NMR chemical shifts of all the hydrogenated carbons could be assigned unambiguously by the HSQC spectrum [18]. In particular, the analysis of the ^{13}C NMR spectrum on the basis of the HSQC correlations clearly showed the occurrence of one olefinic quaternary carbon (δ 141.4), one olefinic methine

Table 3
Effects of compounds **1**–**10** on proliferation of several cancer cell lines.[a].

Compounds	IC_{50} (μM)				
	MCF-7	HT-29	A549	PC3	T98G
1	24	9.2	10	ns[b]	ns[b]
2	34.5	19.8	15.2	25	36
3	ns[b]	30	35	50	70
4	25	43	9.3	ns[b]	ns[b]
5	77	55	11.5	ns[b]	ns[b]
6	70	54	10	ns[b]	ns[b]
7	13	20	9.5	ns[b]	16
8	10	8.3	12	ns[b]	ns[b]
9	ns[b]	ns[b]	60	ns[b]	ns[b]
10	50	18	10	ns[b]	ns[b]
Staurosporine	1.0	1.2	2.0	1.5	4.0
Quercetin	ns[b]	ns[b]	ns[b]	ns[b]	ns[b]

[a] Cell viability was assessed through MTT assay. Cells were treated with different concentrations of compound (1–50 μM) and with solvent alone at 0.1% for 48 h. Staurosporine (1–5 μM) was used as positive control and quercetin (1–50 μM) as negative control. Cytotoxicity, indicated as IC_{50} (μM) for each cell lines, is the concentration of compound which reduced by 50% the optical density of treated cells with respect to untreated cells using the MTT assay. Data represent the mean values of three independent determinations performed in triplicate.

[b] Not significant.

(δ 123.3), three secondary oxygenated carbon (δ 90.0, 79.0 and 77.6) and two carbonyl carbons (δ 215.1 and 220.1), confirmed by the presence in the IR spectrum of an absorption peak at 1754 cm^{-1} (see Table 1). The NMR (^1H, ^{13}C, HSQC, HMBC and DQF-COSY) data of compound **4** were in good agreement with those reported for diginigenin [19]. Thus, the aglycon of **4** was identified as 12α,20α-epoxy-3β-hydroxy-14β,17α-pregn-5-en-11,15-dione. For the sugar portion compound **4** showed in the ^1H NMR spectrum signals corresponding to a secondary methyl at δ 1.32 (3H, *d*, *J* = 6.3 Hz), a methoxy group at δ 3.43 (3H, s) as well as signals for three anomeric protons at δ 4.68 (1H, *dd*, *J* = 9.8, 1.8 Hz), 4.66 (1H, *d*, *J* = 7.9 Hz) and 4.44 (1H, *d*, *J* = 7.9 Hz). All these data indicated that the sugar chain of compound **4** consisted of three sugars, one of them being 2,6-dideoxy sugar. Complete assignments of the ^1H and ^{13}C NMR signals of the sugar portion were accomplished by HSQC, HMBC, DQF-COSY and 1D-TOCSY experiments which led to the identification of one β-diginopyranosyl (δ 4.68) unit and two β-glucopyranosyl (δ 4.66 and 4.44) units (see Table 2). Once again, the sugar sequence and the linkage sites were deduced from HSQC and HMBC experiments. The glycosidation shifts on C-3 (δ 79.0), C-4_{dgn} (δ 74.0) and C-4_{glcI} (δ 80.6) indicated the linkage sites. In the HMBC spectrum key correlation peaks between the proton signal at δ 4.68 (H-1_{dgn}) and the carbon resonance at δ 79.0 (C-3), the proton signal at δ 4.66 (H-1_{glcI}) and the carbon resonance at δ 74.0 (C-4_{dgn}), and the proton signal at δ 4.44 (H-1_{glcII}) and the carbon resonance at δ 80.6 (C-4_{glcI}) were observed. Therefore, compound **4** was deduced to be 12α,20α-epoxy-3β-hydroxy-14β,17α-pregn-5-en-11,15-dione 3-*O*-[β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-diginopyranoside].

The absolute configurations of the sugar units were assigned after acid hydrolysis and identification with authentic samples by TLC, followed by preparative separation of each sugar. The D configuration of galactose, diginose, xylose and glucose was established by comparison of their optical rotation values with those reported in literature [10,11].

Additionally, known compounds belonging to the classes of pregnane and cardenolide glycosides have been isolated. As pregnane glycosides, glucodiginin (**5**) [19], glucodigifolein (**6**) [19] and diginin (**7**) [20] were isolated. As cardenolide glycosides, glucogitoroside (**8**), previously reported from other *Digitalis* spp. [21–23], gitoxigenin 3-*O*-β-D-digitaloside (**9**) [24], digitalin or digitalinum verum (**10**), the latter already reported from the seeds of *D. ciliata* [25], were isolated.

Therefore, along with steroidal saponins and cardenolide glycosides, for the first time pregnane glycosides of the diginigenin series have been isolated from *D. ciliata*.

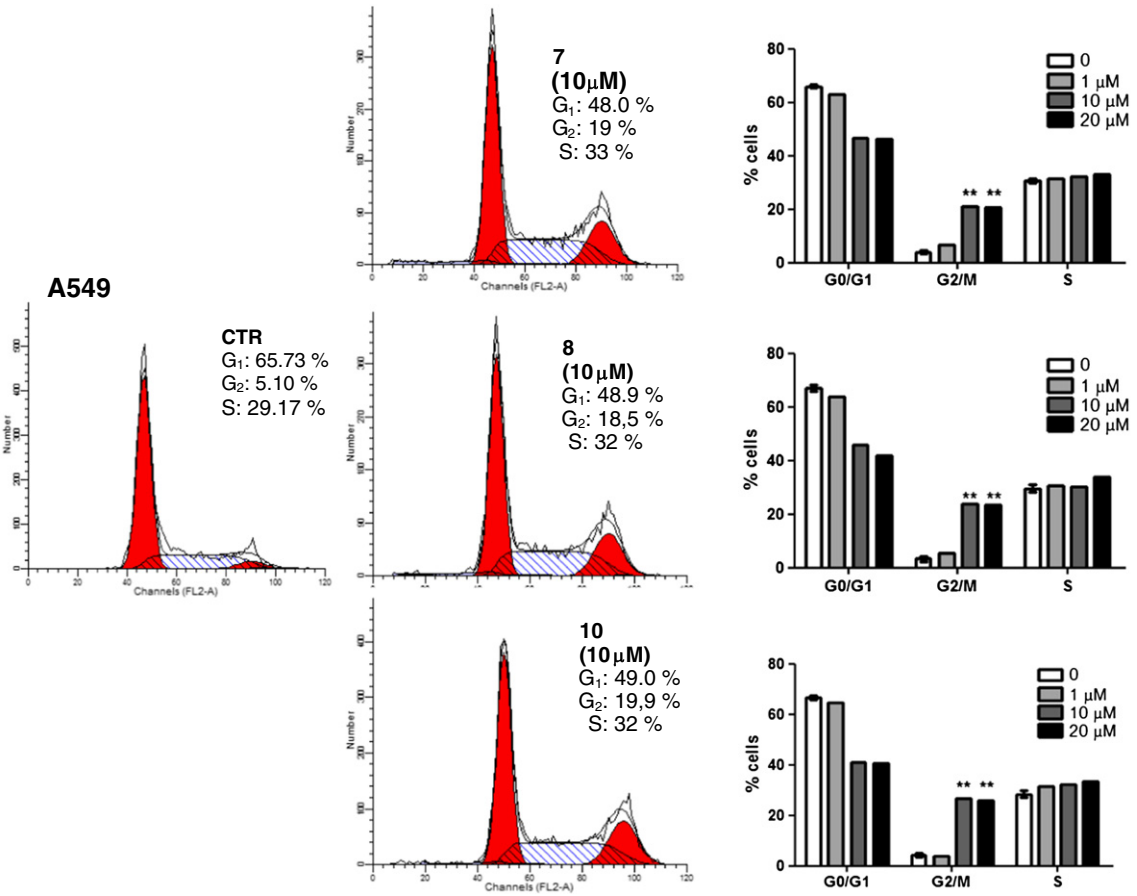

Fig. 2. Representative histograms of cell cycle distribution of A549 cells treated with the compounds **7**, **8** and **10** at different concentrations (1–20 μM) and relative histogram plots of percentage of cells in G_0/G_1, S and G_2/M phases of cell cycle. Data are representative of three experiments performed in triplicate (mean ± SEM). **P < 0.01 vs untreated cells.

On the basis of the cytotoxic activity reported for steroidal, cardenolide and pregnane glycosides [6–9], the effects of compounds **1**–**10** on cell viability of human breast cancer (MCF-7), human glioblastoma (T98G), human lung adenocarcinoma (A549), human colon carcinoma (HT-29), and human prostate cancer (PC-3) cell lines (Table 3) were investigated. Cells were treated with different concentrations (1–50 μM) of compounds **1**–**10** for 48 h and then cell viability was assessed using the MTT assay. DMSO vehicle was used as control (100% cell viability). Compounds **7** and **8** showed IC_{50} values ranging from 8.3 to 20 μM against MCF-7, HT-29 and A549 cell lines. Compound **4** showed an $IC_{50} = 9.3$ μM against A549 cells and also compound **1** showed $IC_{50} = 10$ and 9.2 μM against A549 and HT-29 cells, respectively. Prostate PC3 and glioblastoma T98G cancer cells were not very responsive to the treatment of all analyzed compounds. Staurosporine used as positive control showed IC_{50} values ranging from 1.0 μM in MCF-7 to 4.0 in T98G cells.

Then, the potential antiproliferative effect of the active compounds on MCF-7, HT-29 and A549 cancer cells was investigated.

In order to understand the mechanism involved in cellular death, compounds **1**–**10** were tested as pro-apoptotic agents using propidium iodide staining by flow cytometry as described in methods section. MCF-7, A549 and HT-29 cancer cells were incubated with different concentrations (1–20 μM) of compounds **1**–**10** and of staurosporine (1–5 μM) used as positive control for 24 and 48 h. No significant increase in apoptotic cells after treatments was observed with all the tested compounds, whereas compounds **1** and **2** at 20 μM showed a little necrotic effect (15–20%) on the analyzed cells. Staurosporine used as positive control at 1 μM induced apoptosis (30%) in MCF7, (35%) in A549 and (25%) in HT-29 cancer cells.

Then, the effects of compounds **1**–**10** on cell proliferation by cell cycle analysis of DNA content using flow cytometry were evaluated. MCF-7, A549 and HT-29 cells were incubated with different concentrations (1–20 μM) of compounds **1**–**10** for 48 h. Significant changes in G_2/M cell cycle phase of all analyzed cells were observed, in particular, after treatment with **7**, **8** and **10**. The compounds showed the same behavior by inducing an accumulation of cells in G_2/M phase of cell cycle in all the analyzed cell lines. Compounds **7**, **8** and **10** showed at 10 μM a significant activity in A549 cells (Fig. 2) whereas only **7** and **8** at 10 μM were able to induce an accumulation of cells in G_2/M phase of cell cycle in MCF-7 cells as reported in Fig. 3. Interestingly, compounds **7** and **10** caused a relevant block of HT-29 cancer cells in G_2/M phase of cell cycle. Compound **10** was very active at 10 μM concentration and caused a growth inhibitory effect on HT-29 cells by inducing a strong cell accumulation in phase G_2/M (Fig. 4).

All together the above data indicate that tested compounds are cytostatic compounds effective to reduce cell proliferation of several cancer cells by induction of cell cycle arrest in G_2/M.

4. Conclusion

For the first time in this paper the occurrence of pregnane glycosides of the diginigenin series in *D. ciliata*, along with compounds belonging to the classes of steroidal and cardenolide glycosides is reported. Pregnane, steroidal and cardenolide glycosides, well known for their cytotoxic activities, show an interesting cytostatic activity reducing cell proliferation of several cancer cells by inducing accumulation of the cells in the G_2/M phase of the cell cycle. These results highlight the biological po-

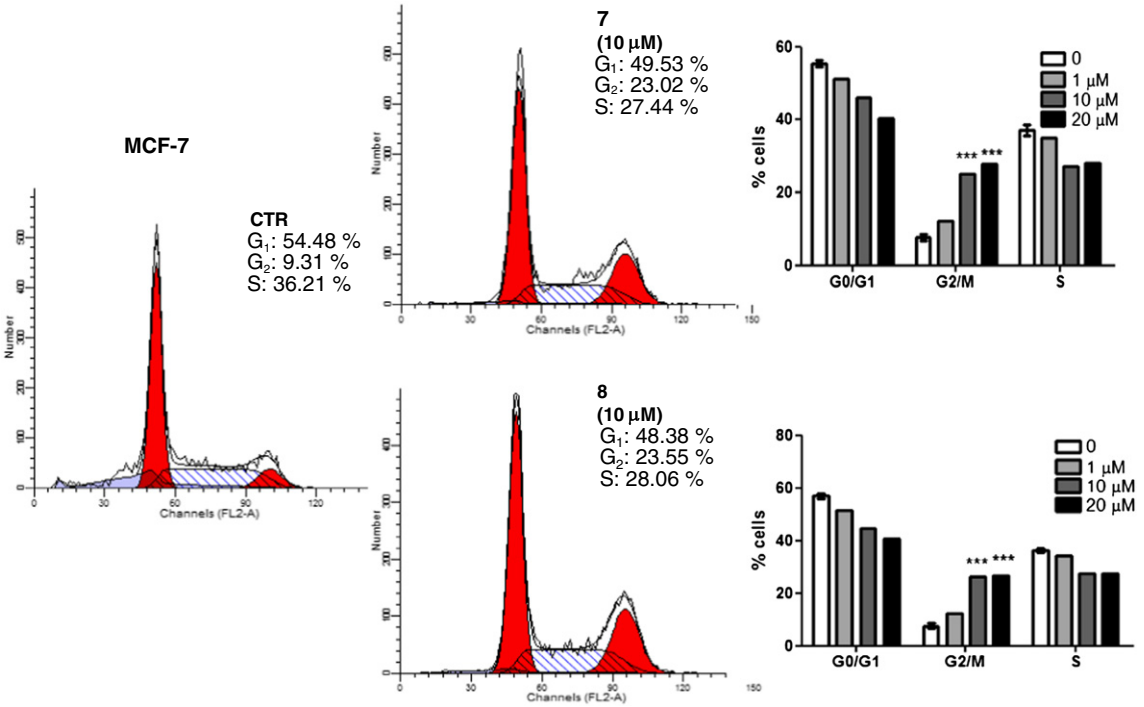

Fig. 3. Representative histograms of cell cycle distribution of MCF-7 cells treated with the compounds **7** and **8** at different concentrations (1–20 μM) and relative histogram plots of percentage of cells in G_0/G_1, S and G_2/M phases of cell cycle. Data are representative of three experiments performed in triplicate (mean ± SEM). ***$P<0.001$ vs untreated cells.

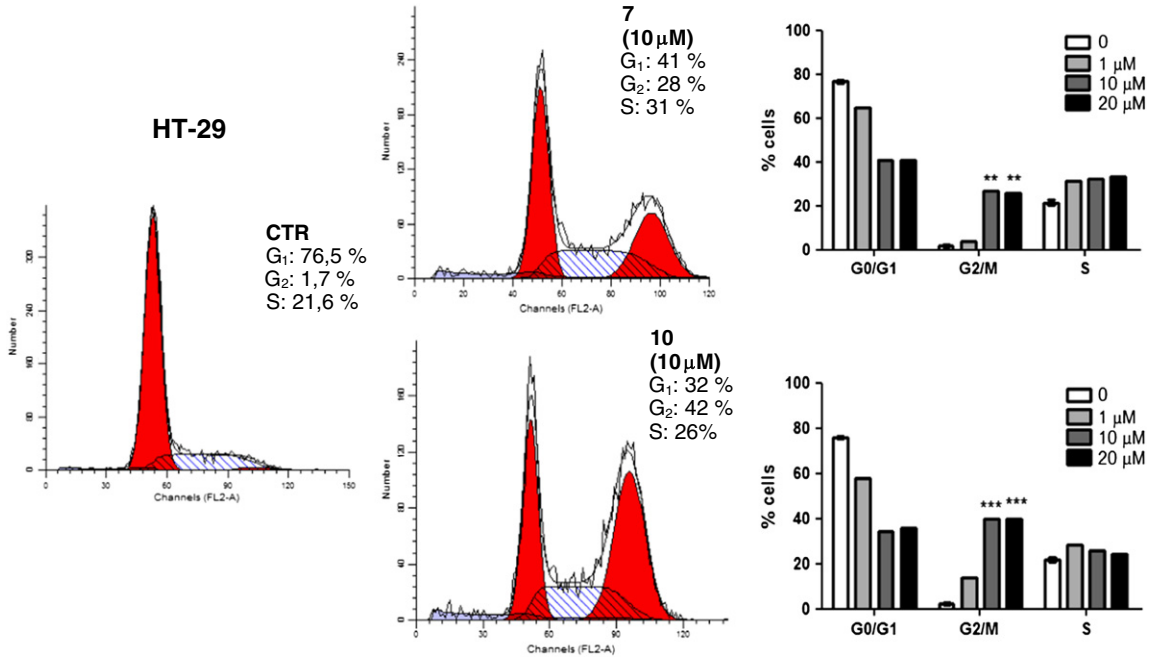

Fig. 4. Representative histograms of cell cycle distribution of HT-29 cells treated with the compounds **7** and **10** at different concentrations (1–20 μM) and relative histogram plots of percentage of cells in G_0/G_1, S and G_2/M phases of cell cycle. Data are representative of three experiments performed in triplicate (mean ± SEM). $^{**}P<0.01$, $^{***}P<0.001$ vs untreated cells.

tential of these classes of metabolites and encourage further studies in order to clarify their antiproliferative mechanism in cancer cell lines.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.fitote.2011.12.020.

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