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Cytotoxic activity of withanolides isolated from Tunisian *Datura metel* L.

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ABSTRACT

Withanolide-type steroids, withametelin Q (**1**) and 12α-hydroxydaturametelin B (**2**) along with three known withanolides, were isolated from leaves of *Datura metel* L. (Solanaceae). The respective structures, characterized mainly by NMR spectroscopy, were identified as (20*R*,22*R*,24*R*)-21,24-epoxy-1α,3β-dihydroxywitha-5,25(27)-dienolide-3-*O*-β-D-glucopyranoside (**1**) and (20*R*,22*R*,24*R*)-12α,21,27-trihydroxy-1-oxowitha-2,5,24-trienolide-27-*O*-β-D-glucopyranoside (**2**). The cytotoxicity of isolated compounds was evaluated against human lung carcinoma cells (A549) and human colorectal adenocarcinoma cells (DLD-1), respectively. Compound **2** exhibited cytotoxicity against A549 and DLD-1 cell lines, with IC_{50} values of 7 and 2.0 μM, respectively. However, for compounds **6** and **7,** cytotoxicities were higher against DLD-1 cells with IC_{50} values of 0.6 and 0.7 μM. Both compounds blocked the cell cycle in the S-phase and induced apoptosis.

1. Introduction

Datura metel L. is a medicinal herb of the Solanaceae family. It has been used in traditional medicine, as a fumigant in bronchial asthma, and for its relaxing effect on the smooth musculature of the bronchi and bronchioles (Smith et al., 1991; Pretorius and Marx, 2006). *Datura* leaves have been used in India and Africa as a treatment for respiratory disorders which have been demonstrated to relieve asthma (Lewis and Elvin-Lewis, 1977). In Tunisia, *Datura* aerial parts are used in folk medicine for their antiasthmatic, antispasmodic and antiparkinsonian properties (Le Floc'h, 1983). The chemical investigations of *Datura* species demonstrated that leaves and seeds especially were rich in alkaloids, including atropine, hyoscyamine, and scopolamine (Berkov et al., 2006; Miraldi et al., 2001). These compounds are included in many official pharmacopoeias because of their anticholinergic activities.

Recently, several pharmacological investigations have been conducted on *Datura*. In fact, different extracts obtained from this genus have been reported to exhibit antimicrobial (Kagale et al., 2004; Eftekhar et al., 2005; Uzun et al., 2004) and antifungal activities (Dabur et al., 2004; Rajesh and Sharma, 2002) as well as hypoglycemic (Krishna Murthy et al., 2004) and antimutagenic properties (Reid et al., 2006).

Cytotoxic activity studies of *D. metel* L. have established the presence of several withanolide-type steroids which inhibit cell growth of human cancer cell lines (Ma et al., 2006; Pan et al., 2007). In the present study, the bioguided fractionation of the MeOH extract from the leaves of *D. metel* L. and the isolation and structure elucidation of two new withanolide-type steroids are reported.

2. Results and discussion

D. metel leaves were extracted using MeOH and MeOH–H_2O (80:20) with a yield of 18%. The cytotoxicity of the MeOH–H_2O extract was assessed against human lung carcinoma (A549), colorectal adenocarcinoma (DLD-1) and normal skin fibroblast (WS1) cell lines. The results were obtained using a resazurin assay and are expressed as the concentration inhibiting 50 percent of cell growth (IC_{50}) (data not shown). The MeOH–H_2O extract was cytotoxic against A549, DLD-1 and WS1 cells with IC_{50} values of 11.9, 3.1 and 7.9 μg mL^{-1}, respectively. A bioguided fractionation of the MeOH–H_2O extract was conducted in order to isolate and identify the compounds responsible for the activity. The MeOH–H_2O extract was successively partitioned with *n*-hexane, dichloromethane and EtOAc. The cytotoxicity of each fraction was again tested. The results showed that the three partitions were cytotoxic against cancer and normal cell lines with IC_{50} values ranging from 1.8 to 11.9 μg mL^{-1} (data not shown).

The EtOAc partition (IC_{50} 3.9–9.5 μg/mL) was selected for further separation because the extraction yield was higher (13.2 g) in comparison with the *n*-hexane (1.4 g) and the dichloromethane partitions (1.1 g). The EtOAc partition was then fractionated on

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Diaion HP-20 resulting in five new fractions named A, B, C, D and E and all were tested for their biological activities. The results showed that fraction B exerted highest cytotoxicity against A549 (IC_{50} = 5.3 μg mL^{-1}) and DLD-1 (IC_{50} = 2 μg mL^{-1}) cells, compared to fractions A, C, D and E. Fraction B (3.5 g) was separated by repeated column chromatography to afford 9 fractions (B1–B9). Fractions B1–B6 showed highest cytotoxicity with IC_{50} values ranging from 0.5 to 5.5 μg mL^{-1} (data not shown). These bioactive fractions were submitted to fractionation by combining column chromatography and preparative reversed-phase HPLC to give five pure compounds. Three known compounds daturametelin B (**6**), withametelin (**7**) and daturametelin A (**8**), were identified by NMR spectroscopic analysis and comparison with literature data (Shingu et al., 1987). Unknown compounds **1** and **2** were identified using NMR (Tables 1 and 2) and mass spectroscopy as described below. The structures of compounds were presented in Fig. 1.

Compound **1** was obtained as a white amorphous solid with a molecular formula of $C_{34}H_{50}O_{10}$ as determined from its HRMS pseudomolecular ion peak at *m/z* 641.3295 (calcd. 641.3302 for $[M+Na]^+$). IR absorption bands were observed at 3383, 2938, 1725, 1223 and 1066 cm^{-1} suggesting the presence of an alcohol and an α,β-unsaturated lactone. The ^{13}C and DEPT-135 NMR spectra of **1** showed peaks attributed to six quaternary, 14 methine, 11 methylene, and three methyl carbons. Detailed analysis of ^1H and HSQC spectra confirmed the presence of a trisubstituted double bond at δ_C 139.1 (C-5), 125.4 (C-6) and δ_H 5.52 (1H, m, H-6), a terminal olefin at δ_H 6.09 and 6.67 (each 1H, s, H_2-27) and three tertiary methyl groups at δ_H 0.73 (3H, s, H_3-18), 1.02 (3H, s, H_3-19) and 1.43 (3H, s, H_3-28), almost identical to those of withametelin (**7**) (Shingu et al., 1987; Oshima et al., 1987). The difference between compounds **1** and **7** was established by ^1H–1H COSY coupling of two oxymethine protons at δ_H 3.81 (1H, br s, H-1) and 4.05 (1H, tt, *J* = 4.8 and 11.1 Hz) with a methylene group at δ_H 1.83 (1H, m, H-2_{ax}) and 2.13 (1H, m, H-2_{eq}) suggesting the presence of 1,3-dihydroxy groups. The latter was located in ring A from the long-range correlations between H_3-19 and δ_C 73.6 (C-1), 42.6 (C-9), 42.8 (C-10) and 139.1 (C-5). The sugar moiety, characterized by COSY, was determined to be β-glucose as supported by the large coupling constant of the anomeric proton at δ_H 4.37 (1H, d, *J* = 7.8 Hz, H-1′) (Agrawal, 1992). Furthermore, long-range correlations between H-1′ and C-3 and between H-3 and C-1′ indicated that the β-glucosyl unit was attached to C-3.

The ^1H and ^{13}C NMR chemical shift of ring C and D and of the lactonic side-chain are almost identical to withametelin (**7**) (Oshima et al., 1987), also isolated in this work. This comparison is important since the absolute stereochemistry of compound **7** was indirectly determined by a X-ray crystallographic analysis (Shingu et al., 1990). Thus, the relative configuration of the side-chain, which was difficult to establish from NOESY data, could be deduced to be the same as the compound **7**. Furthermore,

Table 1
^1H NMR spectroscopic data of compounds **1**–**5**.

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]
1	3.81, *br s*		3.85, *br s*		7.12, *d* (7.6)
2a	1.83, *m*	5.82, *ddd* (1.0, 3.0, 9.9)	1.75, *m*	5.88, *ddd* (0.8, 2.9, 9.9)	6.98, *t* (7.5)
2b	2.13, *m*		2.09, *m*		
3	4.05, *tt* (4.8, 11.1)	6.91, *ddd* (2.4, 4.9, 9.9)	3.99, *tt* (4.6, 11.5)	6.82, *ddd* (2.4, 4.9, 9.9)	6.93, *d* (6.8)
4a	2.31, *m*	2.89, *dd* (4.8, 21.3)	2.30, *m*	2.86, *dd* (5.1, 21.5)	
4b	2.47, *m*	3.35, *m*	2.39, *m*	3.30, *m*	
6a	5.52, *m*	5.63, *br d* (6.0)	5.60, *br d* (5.4)	5.59, *br d* (5.7)	2.62, *m*
6b					2.77, *m*
7a	1.61, *m*	1.63, *m*	1.62, *m*	1.64, *m*	1.38, *m*
7b	1.97, *m*	2.02, *m*	2.01, *m*	2.00, *m*	2.00, *m*
8	1.50, *m*	1.48, *m*	1.51, *m*	1.46, *m*	1.36, *m*
9	1.74, *m*	2.00, *m*	1.65, *m*	2.03, *m*	2.27, *m*
11a	1.47, *m*	1.81, *m*	1.47, *m*	1.80, *m*	1.51, *m*
11b	1.57, *m*	2.46, *dt* (3.3, 14.2)	1.53, *m*	2.42, *dt* (3.2, 14.5)	2.34, *m*
12a	1.42, *m*	4.05, *br s*	1.45, *m*	4.12, *br s*	1.57, *m*
12b	1.96, *m*		1.95, *m*		2.09, *m*
14	1.19, *m*	1.72, *m*	1.17, *m*	1.77, *m*	1.35, *m*
15a	1.19, *m*	1.24, *m*	1.18, *m*	1.22, *m*	1.31, *m*
15b	1.72, *m*	1.75, *m*	1.71, *m*	1.72, *m*	1.82, *m*
16a	1.42, *m*	1.46, *m*	1.40, *m*	1.43, *m*	1.40, *m*
16b	1.83, *m*	1.79, *m*	1.75, *m*	1.72, *m*	1.85, *m*
17	1.81, *m*	2.15, *m*	1.77, *m*	2.18, *m*	1.87, *m*
18	0.73, *s*	0.82, *s*	0.69, *s*	0.76, s	0.75, s
19	1.02, *s*	1.25, *s*	1.03, *s*	1.22, s	2.18, s
20	1.81, *m*	1.83, *m*	1.86, *m*	1.85, *m*	1.85, *m*
21a	3.67, *dd* (1.6, 13.2)	3.86, *m*	3.73, *dd* (3.0, 13.2)	3.85, *dd* (3.0, 12.6)	3.70, *dd* (1.8, 13.3)
21b	3.94, *d* (13.2)	4.05, *d* (12.0)	3.91, *br d* (13.2)	4.10, *m*	3.97, *d* (13.3)
22	4.70, *br s*	4.51, *dt* (3.4, 13.0)	4.66, *br s*	4.45, *m*	4.72, *br s*
23a	1.96, *m*	2.35, *dd* (3.2, 18.5)	1.90, *m*	2.19, *m*	1.97, *m*
23b	2.13, *m*	3.06, *dd* (13.0, 18.5)	2.05, *m*	3.21, *dd* (16.9, 18.6)	2.16, *m*
27a	6.09, *s*	4.47, *d* (11.3)	6.02, *s*	4.35, *d* (12.1)	6.10, *s*
27b	6.67, *s*	4.64, *d* (11.3)	6.76, *s*	4.42, *d* (12.1)	6.68, *s*
28	1.43, *s*	2.13, *s*	1.44, *s*	2.03, *br s*	1.45, *s*
1′	4.37, *d* (7.8)	4.32, *d* (7.8)			
2′	3.14, *dd* (7.8, 9.1)	3.16, *dd* (8.0, 8.9)			
3′	3.35, *m*	3.35, *m*			
4′	3.26, *m*	3.28, *m*			
5′	3.26, *m*	3.27, *m*			
6′	3.85, *dd* (1.4, 11.8)	3.67, *dd* (5.1, 11.6)			
	3.64, *m*	3.86, *m*			

[a] Spectra recorded in CD_3OD.
[b] Spectra recorded in $CDCl_3$.

Table 2
^{13}C NMR spectroscopic data of compounds **1**–**5**[a].

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]
1	73.6	206.5	72.9	204.6	124.0
2	37.7	128.4	38.3	127.8	126.3
3	74.9	148.2	66.4	145.9	128.2
4	39.1	34.4	41.4	33.4	137.1
5	139.1	137.4	137.4	135.5	135.9
6	125.4	125.7	125.3	124.7	28.1
7	32.9	31.7	31.7	30.3	29.0
8	33.3	34.5	31.9	33.1	39.6
9	42.6	38.6	41.5	36.7	45.8
10	42.8	51.3	41.6	49.7	141.2
11	21.4	31.3	20.3	31.3	28.1
12	40.8	74.6	39.4	73.5	41.2
13	44.0	47.4	42.8	45.8	44.2
14	57.6	49.1	56.1	47.7	56.5
15	25.3	24.7	24.1	23.5	24.8
16	27.6	26.9	26.6	25.9	27.7
17	48.9	39.7	47.5	37.8	49.0
18	12.9	13.4	12.6	13.1	13.0
19	20.0	19.3	19.4	18.9	19.9
20	41.6	46.0	39.8	44.7	41.6
21	61.6	59.8	60.5	59.4	61.7
22	77.5	79.3	75.6	78.2	77.4
23	33.9	33.7	33.2	32.7	34.0
24	70.9	161.0	69.4	154.5	70.9
25	140.9	123.5	138.9	125.2	140.9
26	167.6	168.6	165.3	167.2	167.6
27	130.6	63.6	130.0	57.5	130.6
28	25.7	20.8	25.6	20.0	25.7
1′	102.7	104.0			
2′	75.2	75.1			
3′	78.1	78.1			
4′	71.8	71.6			
5′	78.0	78.1			
6′	63.0	62.8			

[a] Spectra recorded in CD_3OD.
[b] Spectra recorded in $CDCl_3$.

unambiguous NOESY correlations between H_3-18 and H-20 and H-21 confirmed the β-configuration of C-17. Stereochemistry in rings A and B were linked to rings C and D through NOESY correlation of H_3-19 and H_3-18 with H-8. The configurations of the alcohol groups were determined to be 1α and 3β from the multiplicity of their associated proton signal (broad singlet for H-1 and triplet of triplet for H-3) which was further confirmed from NOESY correlation between H_3-19 and H-1 and between H-3 and H-2α and H-4α.

Acid hydrolysis of compound **1** allowed isolation of (+)-D-glucose (as measured by optical polarimetry from the aqueous fraction) and the rearranged aglycone **5** from the organic phase NMR spectra of compound **5** showed the presence of an aromatic ring which was located in ring A from the HMBC correlations between δ_H 2.18 (3H, s, H_3-19) and δ_C 137.1 (C-4), 135.9 (C-5) and 128.2 (C-3) and between δ_H 7.12 (1H, d, J = 7.6 Hz, H-1) and δ_C 45.8 (C-9). This result could be explained by acid-catalyzed dehydration of **1** followed by rearrangement of ring A. A similar rearrangement was observed when 5α,6α-epoxy-3β-methanesulfonoxyandrostan-17-one was treated with HBr in acetic acid (Hanson and Reese, 1983). To the best of our knowledge, aromatic compound **5** has never been described elsewhere. The native aglycone **3** was finally obtained after enzymatic hydrolysis at 37 °C. From the above data, the structure of **1** was identified as (20*R*,22*R*,24*R*)-21,24-epoxy-1α,3β-dihydroxywitha-5,25(27)-dienolide-3-*O*-β-D-glucopy ranoside.

Compound **2** was isolated as a white amorphous solid with a molecular formula of $C_{34}H_{48}O_{11}$ as determined from its HRESIMS pseudomolecular ion at m/z 655.3091 (calcd. 655.3094 for $[M+Na]^+$). Strong bands were observed in IR spectrum at 3369, 2915, 1682, 1663, 1076 cm^{-1} suggesting the presence of alcohols and α,β-unsaturated ketones. The ^{1}H and ^{13}C NMR spectra were found to be almost identical to those of daturametelin B (**6**), also isolated during this work. Careful analysis of COSY, HSQC and HMBC spectra allowed determination of an additional alcohol at C-12. This was supported by HMBC correlation between H_3-18 and C-12 at δ_C 74.6. The stereochemistry at position 12 was determined to be α since H-12 at δ_H 4.05 was a broad singlet. Acid hydrolysis of compound **2** afforded (+)-D-glucose (as measured by optical polarimetry from the aqueous fraction) and the aglycone **4**, which has never been described in the literature. Furthermore, long-range correlation between H-1′ and C-26 and between H_2-27 and C-1′ indicated that the β-glucosyl unit was attached to C-27. Therefore, the structure of **2** was elucidated as (20*R*,22*R*,24*R*)-12α,21,27-trihydroxy-1-oxowitha-2,5,24-trienolide-27-*O*-β-D-gluc opyranoside.

The cytotoxicity of compounds **1**, **2**, **6–8** was evaluated against A549, DLD-1 and WS1 cell lines and the results are displayed in Table 3. Etoposide and camptothecin were used as positive controls with IC_{50} values respectively of 7 and 0.11 μM for A549 and 4 and 0.31 μM for DLD-1. The results showed that the isolated withanolides were significantly more active against DLD-1 than A549 cells. However, no significant selectivity for cancer cells

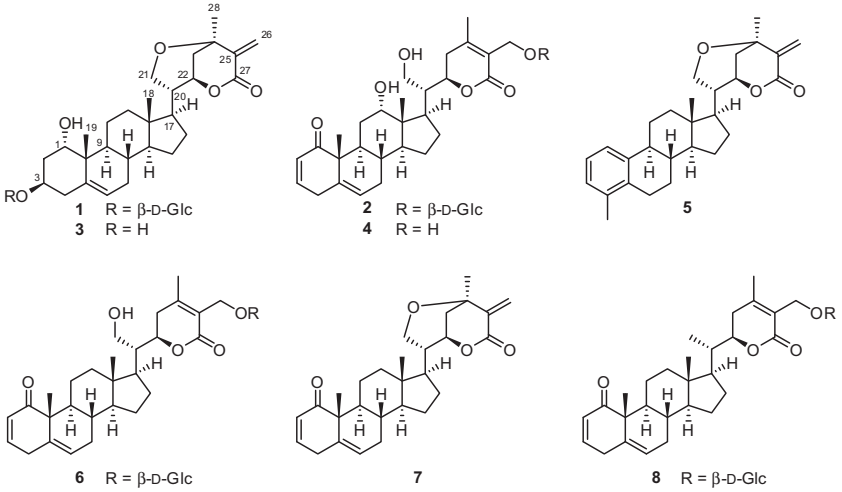

Fig. 1. Structures of compounds **1**–**8**.

Table 3
Cytotoxic activities of compounds **1**, **2** and **6**–**8** against A549, DLD-1 and WS1.

Compounds	IC_{50} (μM)		
	A549	DLD-1	WS1
Daturamalakoside A (**1**)	92 ± 2	17 ± 3	22 ± 2
Daturamalakoside B (**2**)	7 ± 1	2.0 ± 0.3	1.3 ± 0.2
Daturametelin B (**6**)	4.2 ± 0.4	0.6 ± 0.2	1.0 ± 0.1
Withametelin (**7**)	3.5 ± 0.2	0.7 ± 0.1	1.0 ± 0.2
Daturametelin A (**8**)	52 ± 3	24 ± 2	24 ± 3
Etoposide[a]	7.4 ± 0.4	4.0 ± 0.9	21 ± 3
Camptothecin[a]	0.11 ± 0.01	0.31 ± 0.01	0.49 ± 0.04

[a] Control.

was observed in comparison with normal cells. Compounds **6** and **7** were found to be strongly cytotoxic against DLD-1 cells (IC_{50} = 0.6–0.7 μM) and their activities were significantly higher than etoposide and compounds **1**, **2**, **8** and slightly lower than the camptothecin. The results showed that new withanolides **1** and known compound **8** were moderately active against DLD-1 with IC_{50} values of 17 and 24 μM, respectively. Interestingly, the new compound **2** was also found to be strongly active against DLD-1 (IC_{50}: 2 μM) with a cytotoxicity higher than etoposide (IC_{50}: 4 μM). Unfortunately, cytotoxicities of the aglycone of glycosides **1**, **2**, **6** and **8** were not assessed because isolated quantities were not sufficient. The presence of a hydroxyl group on the carbon 21 of compound **6** increased significantly (about 40-fold) the cytotoxicity against DLD-1 cells in comparison with compound **8**. However, the presence of another hydroxyl group on the carbon 12 of compound **2** reduced significantly (about 3-fold) the cytotoxicity in comparison with compound **6**.

As compounds **6** and **7** were strongly cytotoxic against DLD-1 cells, experiments were conducted to evaluate their mode of action. Several studies have shown that withanolides extracted from *Whitania somnifera* induce accumulation of cells in sub-G1 (Senthil et al., 2007) or in G0/G1 after treatment of a new withanolide, tubocapsenolide A, in MDA-MB-231 cells (Chen et al., 2008). Therefore, the effect of compounds **6** and **7** was evaluated on the cell cycle distribution using flow cytometry. Camptothecin used as positive control induces a cell cycle arrest in S-phase and early G2 (Del Bino et al., 1992). The results are presented in Fig. 2A. After 24 h, the viability of cells treated with both withanolides was greater than 85% (data not shown). Surprisingly, the results show after 24 h that, as camptothecin, both withanolides induce an accumulation of DLD-1 cells in the S-phase (43–77% of cells) in comparison with 33% for untreated cells. To our knowledge, this is the first report of an induction of cell cycle arrest in the S-phase by withanolides. A possible explanation is that withanolides possess a lactone ring which could inhibit topoisomerase I activity as camptothecin (Ulukan and Swaan, 2002). However, this assumption will be verified. On the other hand, some withanolides have been shown to induce apoptosis by activating caspases (Senthil et al., 2007) or potentiate apoptosis by inhibiting NF-κB activation (Ichikawa et al., 2006). Moreover, S-phase blockage induced by camptothecin results in apoptosis (Del Bino et al., 1992). Therefore, the effect of compounds **6** and **7** on the induction of apoptosis in DLD-1 cells was assessed using flow cytometry. After 48 h, over 90% of cells treated with compounds **6** and **7** were not viable. The results presented in Fig. 2B shows that both compounds induce preferentially apoptosis at a low dose of 0.1 μM with 93% (compound **6**) and 67% (compound **7**) of cells in sub-G1. In contrast, at a high dose of 5 μM, compounds **6** and **7** induce necrosis with a percentage of cells larger than 80%. Altogether, these results showed that withanolides **6** and **7** induce DLD-1 cell blockage in the S-phase and promoted programmed cell death at low dose and necrosis at high dose.

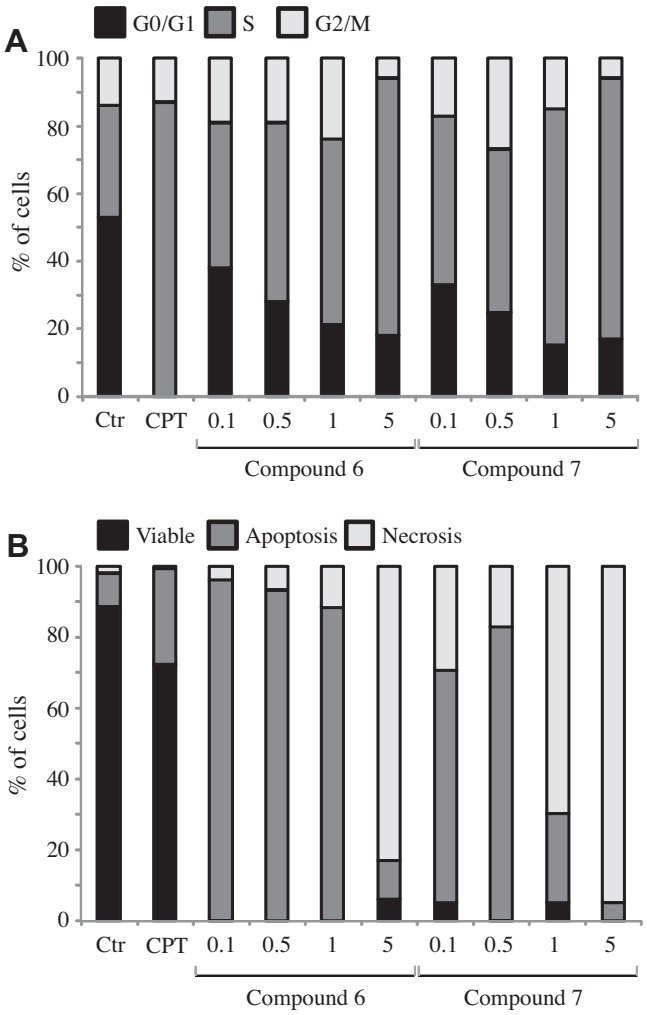

Fig. 2. Withanolide effects on cell cycle after 24 h (A) and on the induction of apoptosis or necrosis after 48 h (B) in DLD-1 cells by flow cytometry analysis. Camptothecin (CPT) 1 μM was used as positive control.

3. Concluding remarks

In conclusion, eight withanolides were isolated from leaves of *D. metel* L. including two new structures (**1** and **2**). Two compounds (**6** and **7**) were found to be strongly active against colorectal cancer cell line, DLD-1. In contrast to others withanolides previously reported in the literature, compounds **6** and **7** blocked the cell in S-phase as camptothecin, a topoisomerase I inhibitor.

4. Experimental

4.1. General experimental procedures

Reversed phase-high performance liquid chromatography analyses were carried out using an analytical Agilent 1100 series with diode array detection and equipped with an Inertsil ODS-C_{18} column (4.6 mm × 250 mm, 5 μm) at room temperature, with MeOH–H_2O (85:15) used as mobile phase. The preparative HPLC instrument used in this study was a preparative Agilent 1100 HPLC Series. Separation was carried out on this system with an Inerstil Prep ODS column (20 mm × 250 mm, 10 μm). MeOH–H_2O (85:15) was used as a mobile phase with flow rate 2 mL min^{-1}. Analytical thin-layer chromatography (TLC) was performed with silica gel 60 F_{254}, 0.25 mm pre-coated TLC plates (Silicycle, Québec,

Canada) using $CHCl_3$/MeOH/H_2O as the mobile phase. Compounds were visualized under visible light after spraying the plates with 20% H_2SO_4 solution in EtOH followed by 1% vanillin in EtOH and heated at 110 °C for 5 min. The 230–400 mesh silica gel (Silicycle, Québec, Canada) was used for column chromatography (CC). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C), equipped with a 5 mm QNP probe. Elucidations of chemical structures were based on analysis of ^{1}H, ^{13}C, COSY, TOCSY, HMBC, HSQC and DEPT-135 experiments. Signals are reported as m (multiplet), s (singlet), d (doublet), t (triplet), dd (doublet of doublet), dt (doublet of triplet), ddd (doublet of doublet of doublet), br s (broad singlet) and coupling constants are reported in hertz (Hz). The chemical shifts are reported in ppm (δ) relative to tetramethylsilane (TMS). The exchangeable O*H* NMR signals appearing sometimes were not listed. Optical rotations were obtained using the sodium D line at ambient temperature on a Rudolph Research Analytical Autopol IV automatic polarimeter. Accurate mass measurements (HRMS) were performed on an LC-MSD-TOF instrument from Agilent Technologies in positive electrospray mode. FTIR spectra were obtained using KBr disks on a Perkin–Elmer Spectrum One Instrument. Chemical reagents were purchased from Sigma–Aldrich Co. Canada or Alfa Aesar Co. and were used as received. The solvents were obtained from VWR International Co. and were used as received.

4.2. Plant material

D. metel L. was collected in August 2008 from its natural habitat in south Tunisia. Leaves were air-dried in darkness at ambient temperature and ground to a fine powder with an electrical mortar. Botanical identification of this species was carried out by Prof. A. Smaoui (Biotechnologic Center in Borj-Cedria Technopark, Tunisia), according to the Tunisian flora (Pottier-Alapetite, 1979). A voucher specimen was deposited in the Aromatic and Medicinal Plants Unit, Biotechnological Center in Borj-Cedria Techno-park, Hammam-Lif, Tunisia (D.m.08003).

4.3. Extraction and isolation

Dried and powdered leaves (0.5 kg) of the plant were extracted with MeOH (3 × 3 L) followed by MeOH–H_2O (80:20) (2 × 2 L), under reflux for 8 h. A sample of 10 mL of the solution of MeOH–H_2O extract was evaporated to dryness to assess the extraction yield to 18% (w,w). The organic phase of the MeOH–H_2O solution was evaporated. The aqueous residue was treated with *n*-hexane (3 × 500 mL) and with CH_2Cl_2 (3 × 500 mL) to remove chlorophyll and other lipophilic constituents. The steroids were finally extracted from the aqueous residue with EtOAc (7 × 500 mL). The EtOAc phase was concentrated to produce a brown powder (13.2 g).

4.4. Bioguided fractionation

The EtOAc fraction was subjected to Diaion HP-20 (Supelco, USA) CC eluting with H_2O–EtOH under increasing percentages of EtOH (from 30% to 100%, v,v) to give five fractions: A (0.82 g), B (3.51 g); C (2.90 g); D (0.26 g); E (0.25 g). Cytotoxicity of all fractions was tested against two cancer cell lines and one normal cell line. The most active fraction B was subjected to open on silica gel CC, eluted with a solvent mixture in various proportions of $CHCl_3$–MeOH–H_2O (55:6:0.5); (50:7:0.5); (50:10:0.5) (50:12:0.5); (50:15:0.5) and finally eluted with MeOH, affording 9 fractions (B1–B9). The B1–B6 fractions showed greatest *in vitro* cytotoxicity against both cancer cell lines. These fractions were further applied to a series of silica gel CC eluting with $CHCl_3$–MeOH–H_2O (50:10:0.5) and with $CHCl_3$–MeOH (80:1), (50:1), (25:1). Finally, isolation was completed by preparative reversed-phase HPLC. New compounds **1** (26.2 mg) and **2** (35.3 mg) were isolated from B4. Daturametelin B (**6**) (100.6 mg) was isolated from B1 and B2 and daturametelin D (**7**) (50.9 mg) was isolated from B3. Daturametelin A (**8**) (16.8 mg) was isolated from B5. All known compounds were identified by comparing spectroscopic data with reported values.

4.4.1. Daturamalakoside A (20R,22R,24R)-21,24-epoxy-1α,3β-dihydroxywitha 5,25(27)-dienolide-3-O-β-D-glucopyranoside (**1**)

White amorphous solid. $[\alpha]_D^{25}$ −35.96 (c = 1, MeOH). IR ν_{max}: 3383, 2938, 2906, 2868, 1725, 1382, 1223, 1066, 1016 cm^{-1}; HRESIMS *m*/*z*: 641.3295. Calcd. for $C_{34}H_{50}O_{10}Na$: 641.3302. For ^{1}H and ^{13}C NMR spectroscopic data (see Tables 2 and 3).

4.4.2. Daturamalakoside B (20R,22R,24R)-12α,21,27-trihydroxy-1-oxo witha-2,5,24-trienolide-27-O-β-D-glucopyranoside (**2**)

Light yellow amorphous solid. $[\alpha]_D^{25}$ +11.90 (*c* = 1, MeOH). IR ν_{max}: 3369, 2915, 1682, 1663, 1398, 1076, 1038 cm^{-1}; HRESIMS *m*/*z*: 655.3092. Calcd. for $C_{34}H_{48}O_{11}Na$: 655.3094. For ^{1}H and ^{13}C NMR spectroscopic data (see Tables 2 and 3).

4.4.3. Daturamalakin A (20R,22R,24R)-21,24-epoxy-1α,3β−dihydroxy witha-5,25(27)-dienolide (**3**)

White amorphous solid. $[\alpha]_D^{25}$ −39.9 (*c* = 0.4, $CHCl_3$). IR ν_{max}: 3400, 2917, 1718, 1565, 1223, 756 cm^{-1}; HRESIMS *m*/*z*: 457.2952. Calcd. for $C_{28}H_{40}O_5$: 457.2954. For ^{1}H and ^{13}C NMR spectroscopic data (see Tables 2 and 3).

4.4.4. Daturamalakin B (20R,22R,24R)-12α,21,27-trihydroxy-1-oxow itha-2,5,24-trienolide (**4**)

Light yellow amorphous solid. $[\alpha]_D^{25}$ +18.1 (*c* = 0.4, $CHCl_3$). IR ν_{max}: 3386, 2922, 1663, 1381, 1078, 754 cm^{-1}; HRESIMS *m*/*z*: 493.2564. Calcd. for $C_{28}H_{38}O_6Na$: 493.2566. For ^{1}H and ^{13}C NMR spectroscopic data (see Tables 2 and 3).

4.4.5. Phenowithanolide (**5**)

White amorphous solid. $[\alpha]_D^{25}$ −3.61 (*c* = 0.5, $CHCl_3$). IR ν_{max}: 2938, 2868, 1720, 1381, 1220, 753, 666 cm^{-1}; HRESIMS *m*/*z*: 420.2731. Calcd. for $C_{28}H_{36}O_3$: 420.2737. The ^{1}H and ^{13}C NMR spectroscopic data (see Tables 2 and 3).

4.5. Acid hydrolysis

In screwed cap vials, 10% of HCl (10 mL) was individually added to compounds **1** and **2** (10 mg each). The resulting solutions heated at 100 °C for 4 h. The aglycones were extracted with $CHCl_3$ (3 × 10 mL), and the aqueous phases containing sugars were neutralized with 10% *N*-methyl-*N*,*N*-di-*n*-octylamine. Monosaccharides were analyzed by TLC with authentic samples using CH_2Cl_2–MeOH–H_2O (50:25:5), spots were visualized using naphthoresorcinol solution in EtOH.

4.6. Enzymatic hydrolysis

Compounds **1** and **2** (20 mg) were dissolved in 50 mL acetate buffer (10 g sodium acetate anhydrous, 1.6 mL glacial acetic acid, 500 mL H_2O and pH 5) and 200 mg of β-glycosidase from almonds (7–8 IU mg^{-1}) was added. The mixture was incubated at 40 °C for 22 h. After the hydrolysis, the reaction mixture was extracted with $CHCl_3$ (3 × 50 mL) (Moreau and Hicks, 2004). The residue (aglycone), after evaporating and drying, was analyzed by NMR spectroscopy.

4.7. Cell culture

The human lung carcinoma A549 (#CCL-185), colorectal adenocarcinoma DLD-1 (#CCl-221) and skin fibroblast WS1 (#CRL-1502) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells lines were grown in minimum essential medium containing Earle's salts (Mediatech Cellgro®, Herndon, USA), supplemented with 10% fetal calf serum (Hyclone, Logan, USA), 1× solution of vitamins, 1× sodium pyruvate, 1× non-essential amino acids, 100 IU of penicillin and 100 μg mL^{-1} of streptomycin (Mediatech Cellgro®). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

4.8. Cytotoxicity assay

Exponentially growing cells were plated at a density of 5×10^3 cells per well in 96-well microplates (BD Falcon) in culture medium (100 μL) and were allowed to adhere for 16 h before treatment. Then, cells were incubated for 48 h in the presence or absence of 100 μL of increasing concentrations of extract, fraction or pure compounds dissolved in culture medium and DMSO. The final concentration of DMSO in the culture medium was maintained at 0.25% (v/v) to avoid toxicity. Cytotoxicity was assessed using the resazurin reduction test (O'Brien et al., 2000). Fluorescence was measured on an automated 96-well Fluoroskan Ascent Fl™ plate reader (Labsystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Cytotoxicity was expressed as the concentration of drug inhibiting cell growth by 50% (IC_{50}).

4.9. Flow cytometry

Cell cycle was evaluated by flow cytometry as described by Pollack and Ciancio (1990). Briefly, an amount of 0.5×10^6 cells were plated in petri dishes and were allowed to adhere for 24 h. After 24 or 48 h of treatment with selected concentration of products, cell pellet resuspended in 50 μL PBS was supplemented with propidium iodide (20 μg mL^{-1}). After 15 min of incubation on ice, cells were fixed in 25% ethanol in PBS. After addition of Hoechst reagent (112 μg mL^{-1}), cells were analyzed using an EPICS ELITE ESP (Beckman-Coulter, Québec, Canada) flow cytometer. Cell cycle analysis was performed with the MulticycleAV software (Phoenix flow system, San Diego, CA).

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References

Agrawal, P.K., 1992. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 31, 3307–3330.
Berkov, S., Zayed, R., Doncheva, T., 2006. Alkaloid patterns in some varieties of *Datura stramonium*. Fitoterapia 77, 179–182.
Chen, W.Y., Chang, F.R., Huang, Z.Y., Chen, J.H., Wu, Y.C., Wu, C.C., 2008. Tubocapsenolide A, a novel withanolide, inhibits proliferation and induces apoptosis in MDA-MB-231 cells by thiol oxidation of heat shock proteins. J. Biol. Chem. 25, 17184–17193.
Dabur, R., Singh, H., Chhillar, A.K., Ali, M., Sharma, G.L., 2004. Antifungal potential of Indian medicinal plants. Fitoterapia 75, 389–391.
Del Bino, G., Bruno, S., Yi, P.N., Darzynkiewicz, Z., 1992. Apoptotic cell death triggered by camptothecin or teniposide. The cell cycle specificity and effects of ionizing radiation. Cell Prolif. 25, 537–548.
Eftekhar, F., Yousefzadi, M., Tafakori, V., 2005. Antimicrobial activity of *Datura innoxia* and *Datura stramonium*. Fitoterapia 76, 118–120.
Hanson, J.R., Reese, P.B., 1983. A deuterium NMR study of 4-methyloestratriene formation. Tetrahedron 24, 3405–3408.
Ichikawa, H., Takada, Y., Shishodia, S., Jayaprakasam, B., Nair, M.G., Aggarwal, B.B., 2006. Withanolides potentiate apoptosis, inhibit invasion, and abolish osteoclastogenesis through suppression of nuclear factor-κB (NF-κB) activation and NF-κB-regulated gene expression. Mol. Cancer Ther. 5, 1434–1445.
Kagale, S., Marimuthu, T., Thayumanvan, B., Nandakumar, R., Samiyappan, R., 2004. Antimicrobial activity and induction of systemic resistance in rice by leaf extract of *Datura metel* against *Rhizoctonia solani* and *Xanthomonas oryzae* pv *Oryzae*. Physiol. Mol. Plant Pathol. 65, 91–100.
Krishna Murthy, B., Nammi, S., Kota, M.K., Krishna Rao, R.V., Koteswara Rao, N., Annapurna, A., 2004. Evaluation of hypoglycemic and antihyperglycemic effects of *Datura metel* (Linn.) seeds in normal and alloxan-induced diabetic rats. J. Ethnopharmacol. 91, 95–98.
Le Floc'h, E., 1983. Contribution à une étude ethnobotanique de la flore tunisienne. Editions Ministère de l'enseignement supérieur et de la recherche scientifique, Tunisie, pp. 402.
Lewis, W.H., Elvin-Lewis, M.P.F., 1977. Medical Botany: Plants Effecting Man's Health. John Wiley and Sons, New York, pp. 90–93.
Ma, L., Xie, C.M., Li, J., Lou, F.C., Hu, L.H., 2006. Daturametelins H, I, and J: three new withanolide glycosides from *Datura metel* L. Chem. Biodiv. 3, 180–186.
Miraldi, E., Masti, A., Ferri, S., Barni Comparini, I., 2001. Distribution of hyoscyamine and scopolamine in *Datura stramonium*. Fitoterapia 72, 644–648.
Moreau, R.A., Hicks, K.B., 2004. The in vitro rates of hydrolysis of phytosterol conjugates and other plant lipids by mammalian digestive enzymes, compared to saponification. Lipids 39, 769–776.
O'Brien, J., Wilson, I., Orton, T., Pognan, F., 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 267, 5421–5426.
Oshima, Y., Bagchi, A., Hikino, H., Sinha, S.C., Sahai, M., Ray, B., 1987. Withametelin, a hexacyclic withanolide of *Datura metel*. Tetrahedron 28, 2025–2027.
Pan, Y., Wang, X., Hu, X., 2007. Cytotoxic withanolides from the flowers of *Datura metel*. J. Nat. Prod. 70, 1127–1132.
Pollack, A., Ciancio, G., 1990. Cell cycle phase-specific analysis of cell viability using Hoechst 33342 and propidium iodide after ethanol preservation. Methods Cell Biol. 33, 19–24.
Pottier-Alapetite, G., 1979. Flore de la Tunisie. Angiospermes, Dicotylédones Dialypétales. vol. I, Imprimerie officielle de la république Tunisienne, Tunis, 254pp.
Pretorius, E., Marx, J., 2006. *Datura stramonium* in asthma treatment and possible effects on prenatal development. Environ. Toxicol. Pharmacol. 21, 331–337.
Rajesh, G., Sharma, L., 2002. Studies on antimycotic properties of *Datura metel*. J. Ethnopharmacol. 80, 193–197.
Reid, K.A., Maes, J., Maes, A., Van Staden, J., De Kimpe, N., Mulholland, D.A., Verschaeve, L., 2006. Evaluation of the mutagenic and antimutagenic effects of South African plants. J. Ethnopharmacol. 106, 44–50.
Senthil, V., Ramadevi, S., Venkatakrishnan, V., Giridharan, P., Lakshmi, B.S., Vishwakarma, R.A., Balakrishnan, A., 2007. Withanolide induces apoptosis in HL-60 leukemia cells via mitochondria mediated cytochrome c release and caspase activation. Chem. Biol. Inter. 167, 19–30.
Shingu, K., Kajimoto, T., Furusawa, Y., Nohara, T., 1987. The structures of daturametelin A and B. Chem. Pharm. Bull. 35, 4359–4361.
Shingu, K., Furusawa, Y., Marubayashi, N., Ueda, I., Yahara, S., Nohara, T., 1990. The structure of daturametelin D. Chem. Pharm. Bull. 38, 2866–2867.
Smith, E.A., Meloan, C.E., Pickell, J.A., Oehme, F.W., 1991. Scopolamine poisoning from home made 'moon flower wine'. J. Anal. Toxicol. 15, 216–219.
Ulukan, H., Swaan, P.W., 2002. Camptothecins: a review of their chemotherapeutic potential. Drugs 62, 2039–2057.
Uzun, E., Sariyar, G., Adsersen, A., Karakoc, B., Otük, G., Oktayoglu, E., Pirildar, S., 2004. Traditional medicine in Sakarya province (Turkey) and antimicrobial activities of selected species. J. Ethnopharmacol. 95, 287–296.