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Withanolides from *Withania aristata* and their cytotoxic activity

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ABSTRACT

Seven new withanolides (**1**–**7**), along with three known ones (**8**–**10**), were isolated from the leaves of *Withania aristata*. Their structures were elucidated on the basis of spectroscopic analysis, including 2D NMR experiments and spectrometric techniques, and the absolute configuration of **1** and **2** was established by CD analysis. In the search for new cytotoxic compounds from *Withania* species, the isolated compounds **1**–**9**, along with two derivatives, were assayed for their cytotoxicity against HeLa, MCF-7 and A-549 human tumor cell lines. Derivative (4*S*,20*R*,22*R*)-27-acetoxy-4-*p*-bromobenzoyloxy-1-oxo-witha-2,5,16,24-tetraenolide (**13**) showed cytotoxicity against all the cell lines assayed with IC_{50} values ranging from 2.8 to 3.6 μM, and (4*S*,20*R*,22*R*)-4,27-diacetoxy-4-hydroxy-1-oxo-witha-2,5,16,24-tetraenolide (**12**) exhibited an IC_{50} value of 5.4 μM on the MCF-7 cell line.

1. Introduction

Withania is a small genus of shrubs belonging to the Solanaceaea family, which are distributed in the East of the Mediterranean area, Macaronesian region and extend to south Asia [1], and some species are well known in traditional medicine. In particular, *Withania somnifera* (L.) Dunal, commonly known as "aswagandha", is one of the major ingredients of ayurvedic preparations prescribed for possessing several properties, including antiinflammatory, antitumor, and antioxidant, and also has been used to treat ulcers, bacterial infections and senile dementia [2]. The therapeutic potential of *Withania* species has been attributed to the presence of withanolides [3], which are steroidal lactones built on an ergostane skeleton of 28 carbons functionalized at carbons 1, 22 and 26. In particular, withaferin A suppresses inflammation [4], and exerts an immunopotentiating effect [5], in addition to its anti-tumorigenesis activity, inducing apoptosis [6] in cancer cells and inhibiting angiogenesis [7]. More recently, it has been reported as a treatment for central nervous system disorders [8].

In the Canary Islands, the genus *Withania* is represented by three species: *W. somnifera* (L.) Dunal, *Withania frutescens* (L.) Pauqui and *Withania aristata* (Aiton) Pauqui. *W. aristata*, the only endemic species [9], is widely used in folk medicine as antitumoral, antispasmodic, antirheumatic, for eye and otitis problems, as well as for insomnia [10] and urinary pathologies [11]. However, the only reports in the literature of phytochemical studies on *W. aristata* are of the isolation of six withanolides [12–14], and of their cytotoxic [15] and diuretic [14] activities, along with other constituents [16].

As part of an ongoing phytochemical investigation into endemic species of the Canary Islands, we report herein on the isolation of seven new withanolides (**1**–**7**) from the leaves of *W. aristata*. Their structures were determined on the basis of spectrometric and spectroscopic data by application of 1D and 2D NMR techniques, including COSY, HSQC, HMBC, and ROESY experiments. The absolute configuration of **1** and **2** was established by analysis of their CD curves and thus of the *p*-bromobenzoyl derivative of **1** (**13**). In addition, three known withanolides were isolated and identified as 4β,17α,27-trihydroxy-1-oxo-witha-2,5,24-trienolide (**8**) [17], 4β,27-dihydroxy-1-oxo-witha-2,5,24-trienolide (**9**) [18] and 4β-hydroxy-1-oxo-witha-2,5,24-trienolide (**10**) [19] by comparison of their spectral data with those reported in the literature. Isolated compounds **1**–**9** and derivatives **12** and **13** were assayed for their cytotoxicity against HeLa (carcinoma of the cervix), A-549 (lung carcinoma), and MCF-7 (breast adenocarcinoma) human cell lines. The evaluated derivatives **12** and **13** exhibited the highest potency, followed by compounds **1**–**4** that showed only weak cytotoxicity.

2. Experimental

2.1. General methods

Optical rotations were measured on a Perkin Elmer 241 automatic polarimeter in $CHCl_3$ at 20 °C and the $[\alpha_D]$ are given in

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Table 1
^{1}H (400 MHz), and ^{13}C (100 MHz) NMR data (δ, $CDCl_3$, J values in Hz in parentheses) of **1**–**4**.

No.	**1**		**2**		**3**		**4**	
	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]
1		203.5 s		203.2 s		201.9 s		203.1 s
2	5.91 d (10.1)	128.7 d	5.95 d (10.0)	128.9 d	6.67 d (10.3)	140.0 d	5.96 d (10.1)	128.8 d
3	6.76 dd (4.4, 10.1)	143.0 d	6.77 dd (4.5, 10.0)	142.5 d	6.73 d (10.3)	138.8 d	6.78 dd (4.5, 10.1)	142.7 d
4	4.61 d (4.4)	69.0 d	4.64 d (4.5)	69.3 d		187.7 s	4.64 d (4.5)	69.1 d
5		138.6 s		138.8 s		139.5 s		138.8 s
6	5.90 br s	130.7 d	5.92 br s	130.9 d	6.86 dd (2.2, 10.3)	137.5 d	5.95 br s	130.4 d
7	1.43[b], 2.08 m	31.1 t	1.68, 2.01 m	31.1 t	1.86, 2.32 m	30.5 t	1.72, 2.13 m	30.7 t
8	1.89 m	31.0 d	1.91 m	30.8 d	1.72 m	30.0 d	1.66 m	31.2 d
9	1.64 m	43.2 d	1.65 m	42.5 d	1.87 m	42.9 d	1.65[b] m	42.5 d
10		49.3 s		49.3 s		51.3 s		49.1 s
11	1.57, 2.21 m	22.5 t	1.44, 2.23 m	22.6 t	1.56, 2.34 m	22.0 t	1.67, 2.30 m	22.9 t
12	1.50, 1.71m	34.3 t	1.52, 1.73 m	34.3 t	1.96, 2.15 m	31.0 t	1.71, 2.34 m	36.8 t
13		46.6 s		46.6 s		46.8 s		44.4 s
14	1.43[b] m	56.9 d	1.43 m	57.0 d	1.51 m	56.7 d	1.65[b] m	52.5 d
15	2.13 m	30.5 t	1.56, 2.10 m	30.5 t	1.57, 1.78 m	34.2 t	1.65[b] m	36.0 t
16	5.51 br s	124.3 d	5.53 br s	124.0 d	5.55 br s	124.3 d	4.72 br s	71.5 d
17		155.2 s		155.5 s		155.2 s		151.3 s
18	0.81 s	16.2 q	0.85 s	16.2 q	0.85 s	16.2 q	0.93 s	16.3 q
19	1.44 s	22.5 q	1.48 s	22.5 q	1.42 s	23.4 q	1.46 s	22.5 q
20	2.52 m	35.6 d	2.53 m	35.7 d	2.56[b] m	35.7 d		129.2 s
21	1.10 d (6.9)	16.5 q	1.11 d (7.0)	16.4 q	1.13 d (7.1)	16.5 q	1.84 s	12.0 q
22	4.43 m	78.8 d	4.39 m	78.4 d	4.45 m	78.8 d	5.41 dd (3.5, 12.8)	77.8 d
23	2.15, 2.52[b] m	32.6 t	2.09, 2.47 m	32.3 t	2.19, 2.56[b] m	32.6 t	2.26, 2.73 m	34.6 t
24		152.9 s		148.7 s		152.3 s		153.7 s
25		125.3 s		121.8 s		125.5 s		125.0 s
26		166.7 s		166.7 s		166.5 s		166.8 s
27	4.31, 4.36 d_{AB} (12.8)	57.0 t	1.88 s	12.3 q	4.37, 4.42 d_{AB} (11.8)	57.3 t	4.38, 4.43 d_{AB} (12.4)	57.2 t
28	2.02 s	19.7 q	1.93 s	20.2 q	2.03 s	19.7 q	2.03 s	19.6 q

[a] Data are based on DEPT and HSQC experiments.
[b] Overlapping signals.

10^{-1} deg cm^2 g^{-1}. UV spectra were obtained on a JASCO V-560 spectrophotometer, and CD spectra on a JASCO J-600 spectropolarimeter. IR (film) spectra were measured on a Bruker IFS 55 spectrophotometer. NMR experiments were performed on a Bruker Avance 400 spectrometer and chemical shifts are shown in δ (ppm) with tetramethylsilane (TMS) as internal reference. EIMS and HREIMS were recorded on a Micromass Autospec spectrometer, and ESIMS and HRESIMS (positive mode) were measured on a LCT Premier XE Micromass Electrospray spectrometer. Silica gel 60 (15–40) μM for column chromatography, and silica gel 60 F_{254} for preparative thin-layer chromatography plates were purchased from Macherey-Nagel, and Sephadex LH-20 for exclusion chromatography was obtained from Pharmacia Biotech.

2.2. Plant material

Leaves of *W. aristata* were collected in Icod de los Vinos, Tenerife, Canary Islands (Spain), in May 2005. A voucher specimen (TFC 48.068) is deposited in the Herbarium of the Department of Botany, University of La Laguna, Tenerife, and identified by Leticia Rodríguez-Navarro.

2.3. Extraction and isolation

The air-dried powdered leaves of *W. aristata* (1.65 kg) were exhaustively extracted with CH_2Cl_2 in a Soxhlet apparatus and the solvent was evaporated at reduced pressure. The residue (71 g) was fractioned by vacuum-liquid chromatography on silica gel and eluted with hexane/EtOAc mixtures of increasing polarity (from 100:0 to 0:100) affording nine fractions, four of them (VI, VII, VIII, and IX) containing withanolides by previous ^{1}H NMR analysis. Each of these fractions was subjected to column chromatography over Sephadex LH-20 (*n*-hexane/$CHCl_3$/MeOH, 2:1:1), and silica gel (CH_2Cl_2/acetone of increasing polarity). Preparative thin-layer chromatography developed with CH_2Cl_2/acetone (8.5:1.5) was used to purify the new compounds **1** (79.0 mg), **2** (15.0 mg), **3** (8.3 mg), **4** (30.3 mg), **5** (2.3 mg) **6** (2.4 mg) and **7** (3.5 mg), in addition to the known compounds 4β,17α,27-trihydroxy-1-oxo-witha-2,5,24-trienolide (**8**, 9.0 mg), 4β,27-dihydroxy-1-oxo-witha-2,5,24-trienolide (**9**, 9.0 mg) and 4β-hydroxy-1-oxo-witha-2,5,24-trienolide (**10**, 1.2 mg).

2.3.1. (4S,20S,22R)-4,27-Dihydroxy-1-oxo-witha-2,5,16,24-tetraenolide (**1**)

White amorphous solid; $[\alpha]_D^{20}$ = +70.4° (*c* = 0.50, $CHCl_3$); CD (MeOH): λ_{ext} 337 ($\Delta\varepsilon$ = −0.6), 240 ($\Delta\varepsilon$ = +2.0) nm; UV (EtOH) (log ε): λ_{max} 337 (2.1), 235 (3.9) nm; IR (film): ν_{max} 3429, 2970, 2928, 2853, 1689, 1455, 1393, 1013, 755 cm^{-1}; ^{1}H and ^{13}C NMR: data are shown in Table 1; EIMS *m/z* (%): 452 $[M]^+$ (4), 434 (53), 419 (22), 401 (7), 380 (7), 312 (14), 283 (17), 265 (13), 171 (23), 141 (100), 123 (69), 95 (57), 69 (85); HREIMS *m/z*: 452.2578 (calcd. for $C_{28}H_{36}O_5$: 452.2563).

2.3.2. (4S,20S,22R)-4-Hydroxy-1-oxo-witha-2,5,16,24-tetraenolide (**2**)

White amorphous solid; $[\alpha]_D^{20}$ = +40.4° (*c* = 1.20, $CHCl_3$); CD (MeOH): λ_{ext} 339 ($\Delta\varepsilon$ = −0.9), 244 ($\Delta\varepsilon$ = +2.6); UV (EtOH) (log ε): λ_{max} 335 (2.2), 238 (3.8) nm; IR (film): ν_{max} 3430, 2927, 2856, 1690, 1455, 1381, 1242, 1212, 1131, 1014, 757 cm^{-1}; ^{1}H and ^{13}C NMR: data are shown in Table 1; EIMS *m/z* (%): 436 $[M]^+$ (3), 418 (9), 403 (8), 345 (1), 293 (9), 265 (7), 171 (11), 125 (100), 97 (20); HREIMS *m/z*: 436.2643 (calcd. for $C_{28}H_{36}O_4$: 436.2614).

2.3.3. (20S,22R)-27-Hydroxy-1,4-dioxo-witha-2,5,16,24-tetraenolide (**3**)

White amorphous solid; $[\alpha]_D^{20}$ = +26.6 (*c* = 0.83, $CHCl_3$); UV (EtOH) (log ε): λ_{max} 218 (4.3) nm; IR (film): ν_{max} 3446, 2927, 1694, 1625, 1458, 1393, 1271, 1129, 1022, 755 cm^{-1}; ^{1}H and ^{13}C NMR:

Table 2
^{1}H (400 MHz), and ^{13}C (100 MHz) NMR data (δ, $CDCl_3$, J values in Hz in parentheses) of **5**–**8**.

No.	**5**		**6**		**7**		**8**	
	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]
1		204.2 s		202.1 s		203.7 s		203.2 s
2	5.88 dt (2.6, 10.0)	127.9 d		140.2 d	5.90 dd (2.4, 10.2)	127.6 d	5.95 d (10.0)	128.8 d
3	6.78 dd (2.4, 5.0, 10.0)	145.2 d	6.67 d (10.3)	139.0 d	6.79 dd (2.1, 10.2)	147.5 d	6.77 dd (4.4, 10.0)	142.7 d
4	2.86 dd (5.0, 21.0) 3.32 dd (2.4, 21.0)	33.4 t	6.73 d (10.3)	187.8 s	5.05 br s	67.0 d	4.64 d (4.6)	69.1 d
5		136.0 s		139.6 s		140.2 s		138.6 s
6	5.58 d (8.0)	124.4 d	6.87 dd (2.0, 5.7)	137.4 d	6.05 d (6.1)	121.2 d	5.93 s	130.7 d
7	1.65, 2.00 m	30.5 t	1.85,2.32[b] m	30.7 t	1.65, 2.13 m	30.1 t	1.68, 2.13 m	30.8 t
8	1.70[b] m	31.8 d	1.62 m	30.1 d	1.68 m	32.7 d	1.56 m	32.7 d
9	1.72[b] m	42.8 d	1.87 m	42.7 d	1.66 m	42.8 d	1.59 m	42.2 d
10		50.4 s		51.5 s		50.5 s		49.0 s
11	1.72[b], 2.37m	23.5 t	1.67, 2.31 m	23.5 t	1.57, 2.31 m	22.7 t	1.33, 1.60 m	22.6[b] t
12	1.70[b], 2.33m	37.0 t	1.69, 2.32[b] m	37.1 t	1.62, 1.72 m	31.9 t	1.61, 1.70 m	31.8 t
13		44.5 s		44.9 s		47.7 s		47.6 s
14	1.64 m	52.7 d	1.69[b] m	52.6 d	1.69 m	50.2 d	1.69[b] m	50.2 d
15	1.85, 2.64 m	36.1 t	1.75, 2.36 m	36.0 t	1.27, 1.76 m	23.4 t	1.25, 1.75 m	23.5 t
16	4.72 br s	71.7 d	4.76 br s	71.7 d	1.70, 2.00 m	36.2 t	1.69[b], 1.99 m	36.1 t
17		151.6 s		151.4 s		84.8 s		84.9 s
18	0.96 s	16.4 q	0.97 s	16.6 q	0.87 s	14.7 c	0.84 s	14.7 c
19	1.27 s	18.8 q	1.42 s	23.7 q	1.29 s	19.5 c	1.45 s	22.6[b] c
20		129.3 s		129.5 s	2.37 m	42.4 d	2.34 m	42.4 d
21	1.88 s	12.1 q	1.88 s	12.1 q	1.07 d (7.0)	9.1 c	1.05 d (7.0)	9.1 c
22	5.41 dd (3.6, 12.9)	77.9 d	5.40 dd (3.5, 13.0)	77.8 d	4.70 dt (2.6, 8.8)	79.0 d	4.68 dt (2.8, 8.1)	79.0 d
23	2.26, 2.73 m	34.7 t	2.26, 2.75 m	34.8 t	1.47, 2.57 m	32.6 t	2.51 d (7.8)	32.6 t
24		153.7 s		153.8 s		154.1 s		154.1 s
25		125.1 s		125.1 s		124.9 s		125. 0 s
26		166.9 s		166.7 s		166.9 s		166.9 s
27	4.39, 4.44 d_{AB} (12.6)	57.4 t	4.37, 4.42 d_{AB} (12.2)	57.4 t	4.37, 4.42 d_{AB} (12.5)	57.2 t	4.35, 4.40 d_{AB} (12.6)	57.2 t
28	2.06 s	19.7 q	2.06 s	19.7 q	2.05 s	19.8 c	2.03 s	19.7 c

[a] Data are based on DEPT and HSQC experiments.
[b] Overlapping signals.

data are shown in Table 1; EIMS *m/z* (%): 450 $[M]^+$ (15), 435 (14), 417 (12), 380 (3), 310 (18), 281 (29), 187 (14), 141 (100), 123 (91), 95 (35), 69 (32); HREIMS *m/z*: 450.2406 $[M]^+$ (calcd. for $C_{28}H_{34}O_5$: 450.2406).

*2.3.4. (4S,22R)-4,16β,27-Trihydroxy-1-oxo-witha-2,5,17(20),24-tetraenolide (**4**)*

White amorphous solid; $[\alpha]_D^{20}$ = +14.3 (c = 0.80, $CHCl_3$); UV (EtOH) (log ε): λ_{max} 212 (4.4) nm; IR (film): ν_{max} 3420, 2932, 1688, 1454, 1385, 1292, 1181, 1136, 1016, 755 cm^{-1}; ^{1}H and ^{13}C NMR: data are shown in Table 1; EIMS *m/z* (%): 468 $[M]^+$ (1), 432 (25), 417 (12), 399 (42), 300 (12), 263 (28), 249 (34), 183 (33), 171 (71), 129 (75), 91 (100), 67 (45); HREIMS *m/z*: 468.2512 $[M]^+$ (calcd. for $C_{28}H_{36}O_6$: 468.2502).

*2.3.5. (22R)-16β,27-Dihydroxy-1-oxo-witha-2,5,17(20),24-tetraenolide (**5**)*

White amorphous solid; $[\alpha]_D^{20}$ = +11.7 (c = 0.23, $CHCl_3$); UV (EtOH) (log ε): λ_{max} 219 (3.5) nm; IR (film): ν_{max} 3443, 2923, 2854, 1698, 1457, 1381, 1287, 1135, 1015, 755 cm^{-1}; ^{1}H and ^{13}C NMR: data are shown in Table 2; ESIMS (positive) *m/z* (%): 475 $[M+Na]^+$ (22); HRESIMS *m/z*: 475.2462 $[M+Na]^+$ (calcd. for $C_{28}H_{36}O_5Na$: 475.2460).

*2.3.6. (22R)-16β,27-Dihydroxy-1,4-dioxo-witha-2,5,17(20),24-tetraenolide (**6**)*

White amorphous solid; $[\alpha]_D^{20}$ = +15.1 (c = 0.07, $CHCl_3$); UV (EtOH) (log ε): λ_{max} 218 (4.0) nm; IR (film): ν_{max} 3447, 2926, 2859, 1700, 1452, 1385, 1282, 1137, 1017, 756 cm^{-1}; ^{1}H and ^{13}C NMR: data are shown in Table 2; ESIMS (positive) *m/z* (%): 489 $[M+Na]^+$ (13); HRESIMS *m/z*: 489.2266 $[M+Na]^+$ (calcd. for $C_{28}H_{34}O_6Na$: 489.2277).

*2.3.7. (22R)-4α,17α,27-Trihydroxy-1-oxo-witha-2,5,24-trienolide (**7**)*

White amorphous solid; $[\alpha]_D^{20}$ = −7.3 (c = 0.6, $CHCl_3$); UV (EtOH) (log ε): λ_{max} 217 (4.0) nm; IR (film): ν_{max} 3428, 2930, 2859, 1690, 1463, 1396, 1216, 1133, 1027, 759 cm^{-1}; ^{1}H and ^{13}C NMR: data are shown in Table 2; ESIMS (positive) *m/z* (%): 493 $[M+Na]^+$ (100); HRESIMS *m/z*: 493.2557 $[M+Na]^+$ (calcd. for $C_{28}H_{38}O_6Na$: 493.2566).

*2.3.8. Acetylation of **1***

A mixture of Ac_2O (4 drops), compound **1** (10.0 mg), Et_3N (0.1 mL) in dry Cl_2CH_2 (1.5 mL) was stirred at 0 °C for 2 h. The mixture was evaporated to dryness, and the residue was purified by preparative TLC (*n*-hexane/dioxane, 6:4) to give derivatives **11** (2.5 mg) and **12** (9.5 mg).

*2.3.8.1. (4S,20S,22R)-27-Acetoxy-4-hydroxy-1-oxo-witha-2,5,16,24-tetraenolide (**11**).* White amorphous solid; $[\alpha]_D^{25}$ = +80.1° (c = 0.20, $CHCl_3$); UV (EtOH) (log ε): λ_{max} 338 (2.6), 237 (3.2) nm; IR (film): ν_{max} 3854, 3748, 3673, 2929, 2362, 1701, 1540, 1458, 1397, 1258, 1066, 838 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.87 (3H, s), 1.15 (3H, d, J = 7.0 Hz), 1.46 (1H, m), 1.50 (3H, s), 1.52 (1H, m), 1.56 (1H, m), 1.67 (1H, m), 1.70 (1H, m), 1.73 (1H, m), 1.75 (1H, m), 1.84 (1H, m), 2.08 (3H, s, OAc), 2.10 (3H, s), 2.16 (1H, m), 2.17 (1H, m), 2.18 (1H, m), 2.26 (1H, m), 2.58 (1H, m), 2.60 (1H, m), 4.48 (1H, br d, J = 11.2 Hz), 4.67 (1H, br s), 4.89, 4.94 (2H, d_{AB}, J = 12.3 Hz), 5.57 (1H, s), 5.98 (1H, d, J = 10.2 Hz), 5.99 (1H, br s), 6.79 (1H, dd, J = 4.2, 10.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.4 (q), 16.8 (q), 20.5 (q), 22.7 (q), 22.9 (t), 30.7 (t), 31.3 (d), 31.3 (t), 33.1 (t), 34.6 (t), 35.8 (d), 43.5 (d), 46.9 (s), 49.5 (s), 57.2 (d), 58.0 (t), 69.5 (d), 78.6 (d), 121.9 (s), 124.5 (d), 129.1 (d), 130.9 (d), 139.1 (s), 142.6 (d), 155.5 (s), 156.7 (s), 165.0 (s), 203.3 (s), OAc [22.8 (q), 170.9 (s)]; EIMS *m/z*

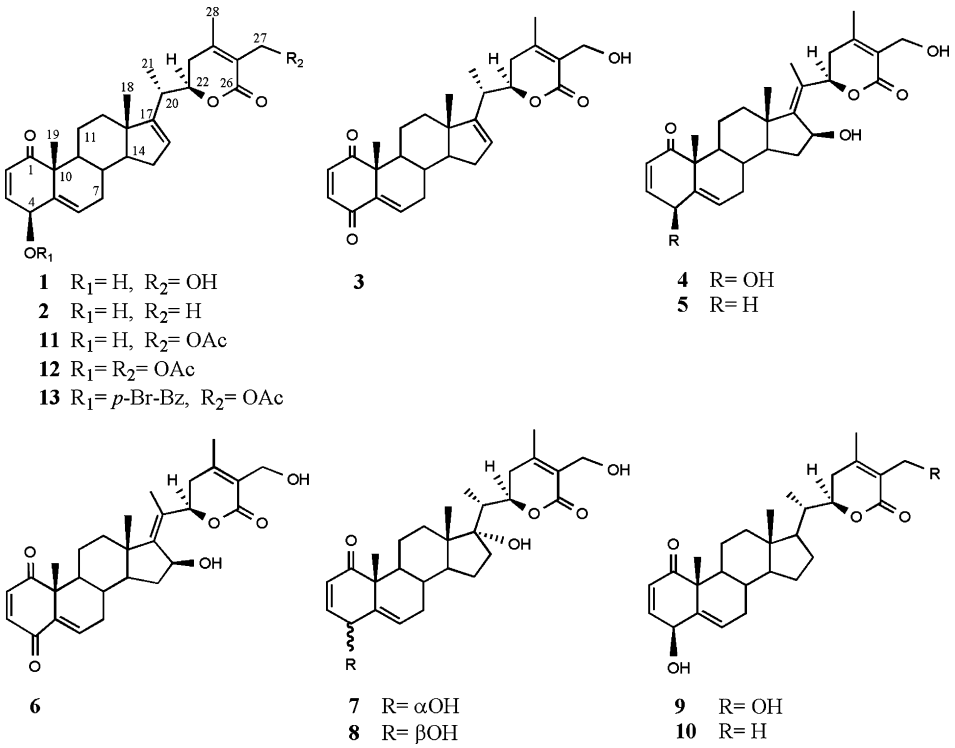

Fig. 1. Structure of natural compounds **1**–**10** and derivatives **11**–**13**.

(%): 476 [M^+-18] (18), 416 (13), 401 (17), 293 (50), 249 (19), 209 (7), 183 (22), 172 (22), 123 (100), 95 (40); HREIMS *m/z*: 476.2578 [M^+-18] (calcd. for $C_{30}H_{36}O_5$: 476.2563).

*2.3.8.2. (4S,20S,22R)-4,27-Diacetoxy-1-oxo-witha-2,5,16,24-tetraenolide (**12**).* White amorphous solid; $[\alpha]_D^{25}$ = +22.4° (*c* = 0.30, $CHCl_3$); UV (EtOH) (log ε): λ_{max} 342 (2.0), 239 (3.7) nm; IR (film): ν_{max} 2926, 2856, 1737, 1514, 1462, 1379, 1235, 1152, 1027, 962, 810, 634; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.87 (3H, s), 1.15 (3H, d, *J* = 7.0 Hz), 1.44 (3H, s), 1.47 (1H, m), 1.55 (1H, m), 1.62 (1H, m), 1.70 (1H, m), 1.71 (1H, m), 1.72 (1H, m), 1.76 (1H, m), 1.94 (1H, m), 2.08 (3H, s, OAc), 2.09 (3H, s), 2.10 (3H, s, OAc), 2.15 (1H, m), 2.17 (1H, m), 2.21 (1H, m), 2.27 (1H, m), 2.56 (1H, m), 2.59 (1H, m), 4.47 (1H, br d, *J* = 11.2 Hz), 4.94, 4.89 (2H, d_{AB}, *J* = 11.8 Hz), 5.56 (1H, br s), 5.81 (1H, d, *J* = 4.6 Hz), 6.04 (1H, d, *J* = 10.0 Hz), 6.13 (1H, d, *J* = 5.0 Hz), 6.72 (1H, dd, *J* = 4.6, 10.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.2 (q), 16.5 (q), 20.4 (q), 21.8 (q), 22.8 (t), 30.7 (t), 31.0 (d), 31.2 (t), 33.0 (t), 34.6 (t), 35.8 (d), 43.5 (d), 46.9 (s), 49.3 (s), 57.1 (d), 58.0 (t), 70.2 (d), 78.8 (d), 121.9 (s), 124.5 (d), 130.9 (d), 133.9 (s), 134.6 (d), 139.8 (d), 155.5 (s), 156.6 (s), 165.0 (s), 203.0 (s), OAc [21.7 (q), 170.2 (s)], OAc [21.8 (q), 170.9 (s)]; EIMS *m/z* (%): 536 [M^+] (1), 476 (17), 416 (13), 401 (24), 327 (7), 293 (47), 249 (19), 197 (5), 171 (38), 159 (17), 123 (100), 95 (41); HREIMS *m/z*: 536.2750 (calcd. for $C_{32}H_{40}O_7$: 536.2774).

*2.3.9. p-Bromobenzoylation of **11***

A mixture of *p*-bromobenzoyl chloride (4.0 mg), compound **11** (2.0 mg), Et_3N (0.05 mL) and DMAP (2.0 mg) in dichloromethane (0.5 mL) was stirred at room temperature for 2 h. The mixture was purified by preparative TLC using dichloromethane/acetone (9:1) to give **13** (2.2 mg).

*2.3.9.1. (4S,20S,22R)-27-Acetoxy-4-p-bromobenzoyloxy-1-oxo-witha-2,5,16,24-tetraenolide (**13**).* White amorphous solid; $[\alpha]_D^{25}$ = +118.2° (*c* = 0.2, $CHCl_3$); CD (MeOH): λ_{ext} 345 (Δε = −1.5), 243 (Δε = +25.1); UV (EtOH) (log ε): λ_{max} 320 (2.7), 240 (4.3) nm; IR (film): ν_{max} 2926, 2856, 1717, 1590, 1457, 1379, 1264, 1096, 1013, 848, 755, 610, 525 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.86 (3H, s), 1.15 (3H, d, *J* = 6.9 Hz), 1.49 (1H, m), 1.55 (3H, s), 1.57 (1H, m), 1.67 (1H, m), 1.72 (1H, m), 1.75 (1H, m), 1.78 (1H, m), 1.79 (1H, m), 1.93 (1H, m), 2.08 (3H, s, OAc), 2.09 (3H, s), 2.14 (1H, m), 2.19 (1H, m), 2.23 (1H, m), 2.33 (1H, m), 2.58 (1H, m), 2.60 (1H, m), 4.47 (1H, br d, *J* = 11.6 Hz), 4.89, 4.94 (2H, d_{AB}, *J* = 12.3 Hz), 5.56 (1H, br s), 6.07 (1H, d, *J* = 4.8 Hz), 6.11 (1H, d, *J* = 9.8 Hz), 6.24 (1H, d, *J* = 5.0 Hz), 6.83 (1H, dd, *J* = 4.8, 9.8 Hz), *p*-Br-OBz [7.61 (2H, d, *J* = 8.3 Hz), 7.90 (2H, d, *J* = 8.3 Hz)]; ^{13}C NMR ($CDCl_3$): δ 16.4 (q), 16.8 (q), 20.5 (q), 22.0 (q), 22.9 (t), 30.8 (t), 31.1 (d), 31.3 (t), 33.1 (t), 34.6 (t), 35.8 (d), 43.6 (d), 46.9 (s), 49.4 (s), 57.0 (d), 58.0 (t), 70.9 (d), 78.5 (d), 121.9 (s), 124.5 (d), 131.3 (d), 134.4 (s), 134.5 (d), 139.5 (d), 155.5 (s), 156.6 (s), 165.0 (s), 202.9 (s), OAc [20.9 (q), 170.9 (s)], *p*-Br-OBz [128.4 (s), 128.9 (s); 131.2 (2 × d), 131.8 (2 × d), 165.1 (s)]; EIMS *m/z* (%): 476 [M^+-p-Br-OBz] (5), 416 (7), 401 (8), 293 (23), 249 (7), 201 (73), 182 (100), 171 (33), 123 (34), 91 (13), 75 (18); HRESIMS (positive) *m/z*: 701.1914 [M+Na]$^+$ (calcd for $C_{37}H_{41}O_7NaBr$: 701.1913).

2.4. Cytotoxicity assay

HeLa (human carcinoma of the cervix), A-549 (human lung carcinoma), and MCF-7 (human breast adenocarcinoma) cell lines were each grown as a monolayer in Dulbecco's modified Eagle's medium, DMEM (Sigma), supplemented with 5% fetal bovine serum (Gibco), and 1% of penicillin–streptomycin mixture (10,000 UI/mL). The cells were maintained at 37 °C in 5% CO_2 and 98% humidity. Cytotoxicity was assessed using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay [20]. Exponentially growing cell suspensions (0.1 ml of 2×10^4 cells/well) were incubated in a microtiter well plate (96-well Iwaki) with the compounds at different concentrations pre-dissolved in DMSO. After 48 h the optical density was measured using a microELISA reader (Multiskan Plus II) at 550 nm after dissolving the MTT formazan with DMSO (150 μL). The percentage viability was plotted against the compound concentration,

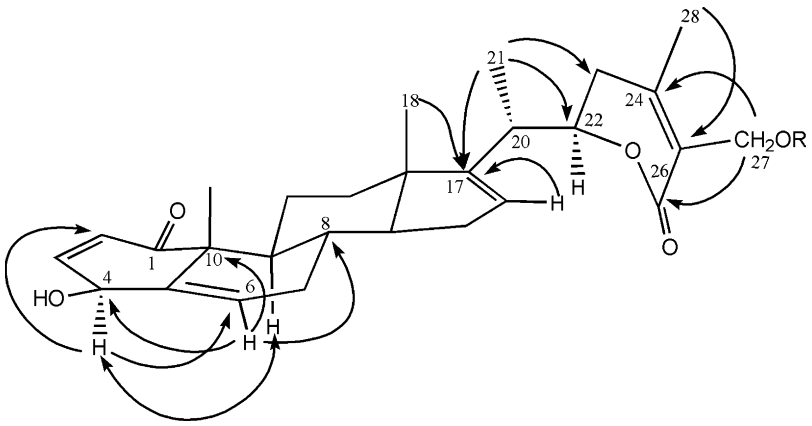

Fig. 2. Selected key HMBC (↷) and ROESY (↶) correlations of **1**.

and the 50% cell viability (IC_{50}) was calculated from the curve. Cytotoxic assays were run in triplicate. The variations were less than 10% and the average value was estimated.

3. Results and discussion

Repeated chromatography of the CH_2Cl_2 extract of the leaves of *W. aristata* on silica gel and Sephadex LH-20 afforded seven new withanolides (**1**–**7**, Fig. 1).

Compound **1** was assigned the molecular formula $C_{28}H_{36}O_5$ by positive high resolution electron ionization mass spectrometry (HREIMS). The IR spectrum revealed the presence of hydroxyl (3429 cm^{-1}) and α,β-unsaturated carbonyl (1689 cm^{-1}) functionalities, and the UV spectrum showed absorption bands at 235 and 337 nm, characteristic of the α,β-unsaturated ketone and α,β-unsaturated δ-lactone systems, respectively, present in most withanolides [21]. The EIMS displayed peaks at *m/z* 452 $[M]^+$, 434 $[M-H_2O]^+$, 312 [M−side chain]$^+$, and a characteristic fragment at *m/z* 141, corresponding to a hydroxy-substituted α,β-unsaturated δ-lactone which originated by the cleavage of the C-20/C-22 bond [22].

The ^1H NMR spectrum of **1** (Table 1) showed signals for two tertiary methyls at δ 0.81 (Me-18) and δ 1.44 (Me-19), a doublet at δ 1.10 (J = 6.9 Hz), suggesting that the secondary methyl at C-21 has the usual α-orientation, and the downfield chemical shift of the C-28 methyl (δ 2.02) indicates that it is located on a double bond. In addition, signals at δ 5.91 (1H, d, J = 10.1 Hz, H-2) and δ 6.76 (1H, dd, J = 4.4, 10.1 Hz, H-3) could be attributed to the most common enone system of withanolides [21]. Moreover, signals of two vinylic protons at δ 5.90 (br s, H-6) and δ 5.51 (br s, H-16), two oxymethine protons at δ 4.61 (d, J = 4.4 Hz, H-4) and δ 4.43 (m, H-22), and a hydroxymethyl group at δ 4.31, 4.36 (d_{AB}, J = 12.8 Hz, H-27) were observed. In the ^1H–^1H COSY experiment of **1**, H-3 correlated with H-2 and the oxymethine proton H-4, indicating the presence of a 4-hydroxy-2-en-1-one unit in the molecule, whereas the H-20 interacted with H-21 and H-22, which in turn is linked to H-23. The H-6 and H-16 also showed vicinal coupling with H-7 and H-15, respectively.

The ^{13}C NMR (Table 1) spectrum indicated the presence of 28 carbon resonances, including four methyl, six methylene, ten methine and eight quaternary carbons. The characteristic downfield signals at δ_C 203.5 and 166.7 were due to the α,β-unsaturated ketone and lactone carbonyl, respectively, and the doublets at δ_C 143.0 and 128.7 were assigned to the vinylic carbons at C-3 and C-2, respectively. The singlets at δ_C 152.9 and 125.3 were attributed to the quaternary vinylic carbons at C-24 and C-25, respectively, and the signals at δ_C 78.8, 69.0 and 57.0 correspond to the oxygenated carbons at C-22, C-4 and C-27, respectively. Moreover, the two doublets at δ_C 130.7 and 124.3 and two singlets at δ_C 138.6 and 155.2 suggested that two additional CH=C are present in the molecule [21].

These data supported that compound **1** is an oxo-withatetraenolide, which presents along with the usual unsaturations at C-2 and C-24, a double bond at C-5, unlike in the more widespread 5β,6β-epoxy withanolide group [21], and an additional one at C-16, which is also unusual in the withanolide skeleton [23]. The Δ^{16}-withanolides have been proposed as precursors not only for 17α-hydroxy- but also for the important group of 17β-hydroxy-withanolides [24].

The regiosubstitution of the different functions was determined by an HMBC experiment (Fig. 2), showing as the most relevant three-bond correlations those of the vinylic proton resonance at δ_H 5.90 (H-6) with the signals at δ_C 69.0 (C-4), 31.0 (C-8) and 49.3 (C-10), and correlation of the proton resonances at δ_H 5.51 (H-16), 0.81 (Me-18), and 1.10 (Me-21) with the signal at δ_C 155.2 (C-17), which confirmed the additional double bonds were sited at C-5 and C-16. Moreover, the oxymethine proton at δ_H 4.61 (H-4) showed correlation with the carbon signals at δ_C 128.7 (C-2), 130.7 (C-6) and 49.3 (C-10), whereas the α,β-unsaturated δ-lactone was located on the side chain due to the cross-peak of the signals at δ_H 1.10 (H-21)/δ_C 78.8 (C-22), δ_H 2.02 (H-28)/δ_C 125.3 (C-25), and δ_H 4.31, 4.36 (H-27)/δ_C 152.9 (C-24) and 166.7 (C-26). The configuration of **1** was established on the basis of the coupling constants, and comparison with values reported in the literature [21], and confirmed by a ROESY experiment (Fig. 2). Accordingly, the structure of **1** was established as 4β,27-dihydroxy-1-oxo-witha-2,5,16,24-tetraenolide.

Compound **2** was isolated as a white amorphous solid with the molecular formula $C_{28}H_{36}O_4$ (HREIMS), and analysis of its spectral data (Table 1) revealed a structure of a 4β-hydroxy-1-oxo-witha-tetraenolide. Therefore, its ^1H NMR spectrum showed signals assignable to an enone group in ring A [δ 5.95 (1H, d, J = 10.0 Hz, H-2) and δ 6.77 (1H, dd, J = 4.5, 10.0 Hz, H-3)], an α,β-unsaturated δ-lactone system (δ 1.88, Me-27 and δ 1.93, M-28), two vinylic protons at δ 5.92 (1H, br s, H-6) and δ 5.53 (1H, br s, H-16), and two oxymethine protons at δ 4.64 (1H, d, J = 4.5 Hz, H-4) and δ 4.39 (1H, m, H-22). The ^{13}C NMR spectrum (Table 1) indicated the presence of 28 carbons resonances, including five methyl, five methylene, ten methine and eight quaternary carbons. These data indicated that compound **2** was related to **1**, the most striking difference observed was the disappearance of the signals for the hydroxymethyl group at C-27 (δ_H 4.31, 4.36, δ_C 57.0) and the presence of an additional methyl group (δ_H 1.88, δ_C 12.3), suggesting compound **2** is the 27-deoxy derivative of **1**. The stereochemistry and regionsubstitution partners were determined by analysis of the ROESY, and HMBC experiments, respectively (Fig. 2). The structure

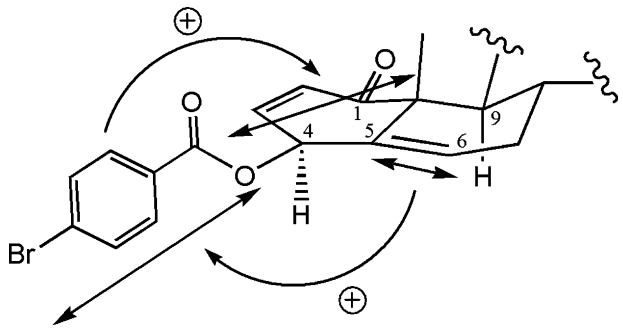

Fig. 3. CD positive pairwise interactions involving the chromophore at C-4, the enone chromophore and the double bond at C-5 for derivative **13**.

of compound **2** was therefore established as 4β-hydroxy-1-oxo-witha-2,5,16,24-tetraenolide.

The absolute configuration of compounds **1** and **2** was determined by means of circular dichroism (CD). Both compounds exhibited a broad negative Cotton effect around 335 nm, corresponding to the n–π* transition of the enone chromophore, as well as a broad positive Cotton effect around 240 nm, assigned to the overlapping of the π–π* transition of the enone and the n–π* transition of the α,β-unsaturated δ-lactone. In addition, the stereochemistry of the lactone ring was determined from the positive Cotton effect around 240 nm (**1**, $\Delta\varepsilon$ = +2.0; **2**, $\Delta\varepsilon$ = +2.6). This positive Cotton effect is in agreement with a 22*R* absolute configuration, as occurred with jaborosolactone A, which was determined by comparison with parasorbic acid, all four exhibiting almost identical intensity at this wavelength [25–27]. In general, withanolides show positive Cotton effects for the n–π* transition of the enone chromophore around 340 nm. However, compounds **1** and **2** exhibits the opposite sign. This could be due to the absence of the 5β,6β-epoxy function present in many withanolides [28]. Although NMR data indicate the 4β configuration at C-4, this was easily confirmed applying the CD exciton chirality method [29] to the *p*-bromobenzoyl derivative of **1** (**13**). Its CD spectrum showed a positive Cotton effect at 243 nm ($\Delta\varepsilon$ = +25.1) in addition to the expected negative Cotton effect at 345 nm ($\Delta\varepsilon$ = −1.5). The positive exciton pairwise interactions involving the ^1L_a band of the *p*-bromobenzoate chromophore and the π–π* transitions of the double bond and the enone chromophores (Fig. 3) are in agreement with a 4*S* absolute configuration. The absolute configuration of withanolides **1** and **2** determined by CD analysis is in agreement with the absolute configuration of withaferin A as determined by X-ray analysis [30].

The structure of compound **3** was defined by spectroscopic data and compared with those of the previously described compound **1**. The HREIMS gave a molecular ion peak at *m/z* 450.2406 providing the molecular formula $C_{28}H_{34}O_5$, which indicated an additional degree of unsaturation with respect to **1**. Its NMR spectra closely matched that of **1**, the main difference being the presence in compound **3** of a ketone signal at δ_C 187.7 (s), as well as the downfield shifts of the signals corresponding to C-2 (δ_C 140.0, d), C-3 (δ_C 138.8, d), C-5 (δ_C 139.5, s) and C-6 (δ_C 137.5, d), together with the absence of the oximethine (δ_H 4.61, d, *J* = 4.4 Hz; δ_C 69.0, d) signals in **1**. Furthermore, a complete set of 2D NMR spectra (COSY, ROESY, HSQC, HMBC) was acquired in order to gain the complete and unambiguous assignment of the ^{1}H and ^{13}C NMR resonances as listed in Table 1, and revealed that **3** was 27-hydroxy-1,4-dioxo-witha-2,5,16,24-tetraenolide.

The structure of compound **4** was established as 4β,16β,27-trihydroxy-1-oxo-witha-2,5,17(20),24-tetraenolide on the basis of spectroscopic data interpretation. Its IR spectrum displayed absorption bands of hydroxyl (3420 cm^{-1}), α,β-unsaturated δ-lactone and α,β-unsaturated ketone (1688 cm^{-1}) functions, these last two moieties are supported by an absorption at λ_{max} 212 nm in the UV spectrum. The molecular formula of **4** was determined to be $C_{28}H_{36}O_6$ by HREIMS at *m/z* 468.2512 (M^+, calcd 468.2502). The NMR spectral data (Table 1) showed four methyl singlets (δ_H 0.93, 1.46, 1.84 and 2.03), three oxygenated methines [δ_H (4.64, d, *J* = 4.5 Hz, H-4; 4.72, br s, H-16; 5.41, dd, *J* = 3.5, 12.8 Hz, H-22)], an oxygenated methylene (δ_H 4.38, 4.43, d_{AB}, *J* = 12.4 Hz, H-27), a trisubstituted double bond [δ_H 5.95, br s, H-6; δ_C 138.8 (C-5), 130.4 (C-6)], a tetrasubstituted double bond [δ_C 151.3 (C-17), 129.2 (C-20)], an α,β-unsaturated δ-lactone [δ_C 153.7 (C-24), 125.0 (C-25), 166.8 (C-26)], and an enone [δ_H 5.96, d, *J* = 10.1 Hz, H-2; δ_H 6.78, dd, *J* = 4.5, 10.1 Hz, H-3; δ_C 203.1 (C-1), 128.8 (C-2), 142.7 (C-3)] moiety. The regiosubstitution was established by an HMBC experiment, and the relative stereochemistry, was defined on the basis of a ROESY experiment. Thus, a cross-peak of H-4 with H-9α and one of H-16 with H-14α confirmed the β stereochemistry of the hydroxyl groups at C-4 and C-16, while correlation of Me-21 with Me-18 and H-12 indicated a *Z* configuration of the Δ^{17} (Fig. 4).

The molecular formula of **5** was determined to be $C_{28}H_{36}O_5$ through ESIMS and NMR data. The strong IR absorption peaks (3443, 1698 cm^{-1}) indicated the presence of hydroxyl, ketone, and lactone groups. The NMR spectroscopic data were similar to those of **4**, except for the presence of a methylene assigned to H_2-4 (δ_H 2.86, *J* = 5.0, 21.0 Hz and 3.32, *J* = 2.4, 21.0 Hz; δ_C 33.4) instead of a methine as in **4** (δ_H 4.64, *J* = 4.5 Hz; δ_C 69.1), suggesting **5** is the 4-dehydroxy derivative of **4**. This conclusion was supported by COSY, HSQC,

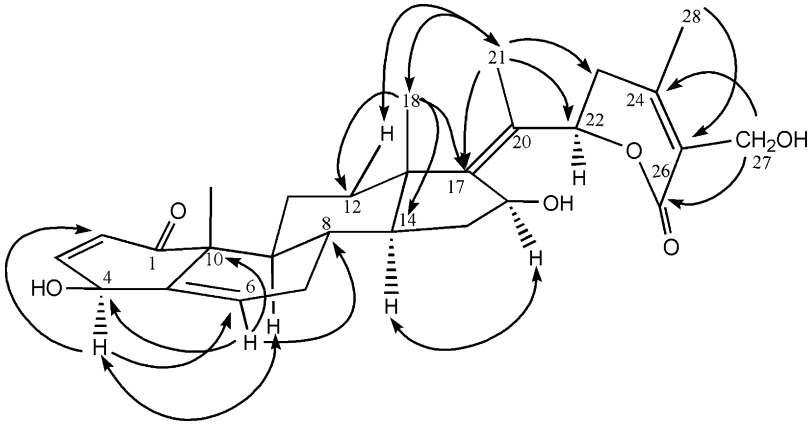

Fig. 4. Selected key HMBC (↷) and ROESY (↶) correlations of **4**.

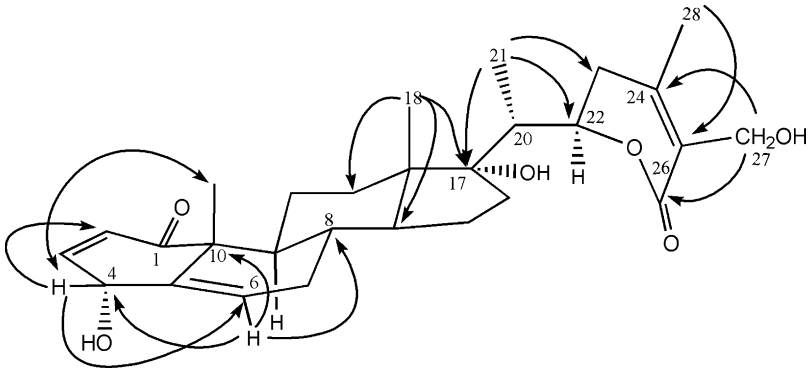

Fig. 5. Selected key HMBC (↷) and ROESY (↶) correlations of **7**.

HMBC and ROESY experiments, and the full assignments of the NMR data are listed in Table 2. Hence, compound **5** was determined to be 16β,27-dihydroxy-1-oxo-witha-2,5,17(20),24-tetraenolide.

Compound **6** was found to have the molecular formula $C_{28}H_{34}O_6$ as determined through HRESIMS and NMR experiments. Its NMR spectroscopic data (Table 2) were similar to those of **4**, except for the presence of a ketone signal at δ_C 187.8, and the absence of the signals of the OH-4 (δ_H 4.64, J = 4.5 Hz; δ_C 69.1) present in **4**. This suggests **6** is the 4-oxo-derivative of **4**; a conclusion supported by 2D experiments. Accordingly, the structure of compound **6** was established as 16β,27-dihydroxy-1-oxo-witha-2,5,17(20),24-trienolide.

Compound **7** showed spectral data resembling those of the known isolated compound **8** [17], and the same molecular formula ($C_{28}H_{38}O_6$) determined by HREISM. The ^{13}C NMR and DEPT spectra (Table 2) were almost identical to those exhibited by **8**, with small differences observed in the chemical shifts of C-2, C-3, C-4, C-6, C-10 and Me-19. Several additional pieces of evidence suggested that **7** was the C-4 epimer of the known isolated **8**. Thus, the ^{1}H NMR spectrum showed the following differences: (i) the absence of the characteristic signals assigned to H-3 (δ_H 6.77) and H-4 (δ_H 4.64) as double doublet and doublet, respectively (ii) the presence of a broad singlet at δ_H 5.05 (H-4) and a double doublet at δ_H 6.79 (H-3); (iii) an upfield shift of the Me-19 (δ_H 1.29 in **7** and δ_H 1.45 in **8**), spectroscopic data that are in agreement with data reported by Hirayama et al. [31] for regioisomeric 4-hydroxymethylnaphthalen-1(4H)-ones. The ROESY experiment (Fig. 5) of **7** confirmed the α-orientation of the 4-hydroxyl group, showing ROE correlation between H-4 and Me-19. Thus, compound **7** was determined to be 4α,17α,27-trihydroxy-1-oxo-witha-2,5,24-trienolide. The occurrence of natural 4α-hydroxy-withanolides is scarce, and there are only a few examples in the literature [32], such as the reports of pubescenol from *Physalis pubescence* [33]. Furthermore, to our knowledge, compound **7** is the first example of a 4α-hydroxy-2-en-1-one withanolide compound.

The detailed ^{1}H and ^{13}C NMR (Table 2) assignments of the one known withanolide 4β,17α,27-trihydroxy-1-oxo-witha-2,5,24-trienolide (**8**) [17], which have not previously reported, were achieved by 1D and 2D techniques including DEPT, HMBC, HSQC, COSY, and ROESY.

The isolated steroids **1**–**9** and derivatives **12** and **13** were tested against a small panel of human cancer cell lines: HeLa (carcinoma of the cervix), MCF-7 (breast adenocarcinoma) and A549 (lung carcinoma) for their *in vitro* cytotoxicity. As summarized in Table 3, derivative **13** showed cytotoxic effect against the tested tumor cell lines (IC_{50} values ranging from 2.8 to 3.6 μM), and **12** exhibited an IC_{50} value of 5.4 μM on the MCF-7 cell line. Furthermore, compounds **1**–**4** showed some degree of activity against HeLa and/or MCF-7 cell lines, with IC_{50} values ranging from 10.3 to 13.9 μM, the other assayed natural compounds (**5**–**9**) being inactive (IC_{50} > 20 μM). These results are in line with previous reports on the relevance of both the 4β-hydroxy-2-en-1-one and the 5β,6β-epoxy moieties for the expression of cytotoxicity [34] in this type of compounds. However, the esterification of the hydroxyl groups at C-4 and C-27 increases the cytotoxicity of the Δ^5-withanolides (**1** versus **12** and **13**).

Table 3
Cytotoxic activity (IC_{50}, μM) of compounds **1**–**9**, **12** and **13**.

Compounds	HeLa	A-549	MCF-7
1	10.8	25.2	13.5
2	11.0	28.4	21.6
3	15.3	35.2	13.9
4	>40	>40	10.3
5	>40	>40	>40
6	>40	>40	>40
7	>40	>40	26.6
8	24.0	>40	27.0
9	19.6	35.7	32.4
12	14.7	17.0	5.4
13	3.5	3.6	2.8
C1[a]	3.2×10^{-3}	0.8×10^{-2}	4.8×10^{-2}
C2[a]	4.1	49.4	5.8

[a] **C1** (actinomycine) and **C2** (mercaptopurine) used as positive controls.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2010.06.001.

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