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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 ($\delta$, $CDCl_3$, $J$ values in Hz in parentheses) of **1**–**4**.

| No. | **1** | | **2** | | **3** | | **4** | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$[a] | $\delta_H$ | $\delta_C$[a] | $\delta_H$ | $\delta_C$[a] | $\delta_H$ | $\delta_C$[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 $\delta$ (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): $\lambda_{ext}$ 337 ($\Delta\varepsilon$ = −0.6), 240 ($\Delta\varepsilon$ = +2.0) nm; UV (EtOH) (log $\varepsilon$): $\lambda_{max}$ 337 (2.1), 235 (3.9) nm; IR (film): $\nu_{max}$ 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): $\lambda_{ext}$ 339 ($\Delta\varepsilon$ = −0.9), 244 ($\Delta\varepsilon$ = +2.6); UV (EtOH) (log $\varepsilon$): $\lambda_{max}$ 335 (2.2), 238 (3.8) nm; IR (film): $\nu_{max}$ 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 $\varepsilon$): $\lambda_{max}$ 218 (4.3) nm; IR (film): $\nu_{max}$ 3446, 2927, 1694, 1625, 1458, 1393, 1271, 1129, 1022, 755 cm$^{-1}$; $^1$H and $^{13}$C NMR: