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Withanolides from *Whitania aristata* and their diuretic activity

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ABSTRACT

Aim of the study: Withania aristata is an endemic plant used traditionally in Canary Islands as a diuretic. In this paper, we report on this pharmacological activity in several extracts of the dry vegetal material collected and the identification and diuretic activity of two withanolides, one of them previously not reported, isolated from the most active fraction.
Material and methods: Four *Whitania aristata* extracts at 100 mg/kg were orally administered to laboratory animals to evaluate their diuretic activity. From the most active fraction, two withanolides were isolated. Both and a mixture of them at 5 and 10 mg/kg were analyzed too as diuretics. Water excretion rate and content of Na^+ and K^+ electrolytes were measured in the urine of saline-loaded animals.
Results: Whitania aristata water fraction, the two withanolides and the mixture of these compounds displayed high diuretic activity, with a significant excretion of sodium and potassium ions in laboratory animals.
Conclusions: This research supports the ethno-medicinal use of *Whitania aristata* as diuretic. This activity seems to be associated to the presence of a new type of natural diuretic agents, such as withaferin A and witharistatin.

1. Introduction

Withania aristata (Ait.) Pauq. (Solanaceae) is an endemic plant from Canary Islands, popularly known as "orobal" or "sáquido". This species has long been used in traditional medicine as a scarring agent, antispasmodic, as well as for rheumatism, eye diseases and otitis, insomnia, constipation and urinary pathologies (Darias et al., 1986, 2001; Pérez-Paz and Hernández Padrón, 1999). From previous studies on the chemical composition of *Whitania aristata*, several withanolides, including withaferin A and withanolide D, have been isolated (González et al., 1972, 1974). Other compounds found were phytosterols, oleoresins and withaminol (Valera and Santos, 2002).

Since the isolation of withaferin A in 1965, over 300 withanolides have been described largely from genera belonging to the Solanaceae (Veleiro et al., 2005). Recently, some of the withanolides isolated from these plants have shown interesting biological activities such as antiarthritic, anticholinesterase, antioxidant, immunoprotective, antitumor, trypanocidal, antimalarial and leishmanicidal, etc. (Jayaprakasam et al., 2003, 2004; Choudhary et al., 2005; Abe et al., 2006; Bani et al., 2006; Cardona et al., 2006; Ichikawa et al., 2006; Khanna et al., 2007; Malik et al., 2007; Muregi et al., 2007). However, till now, no studies on the diuretic activity have been found with these steroidal compounds.

On the other hand, previous studies by our group have shown that the aqueous and methanol extracts, and fractions obtained from the methanol extract, produced important diuretic activity in rats (Martín-Herrera et al., 2007, 2008). So, in order to identify the compounds responsible for this activity, the whole dry plant material collected was submitted to successive extractions with different polar solvents, and two withanolides, one of them not reported previously, were isolated from the fraction that had shown the most diuretic activity. These compounds were also tested to establish their potential diuretic activity.

2. Material and methods

2.1. Plant material

The leaves of flowering and immature fruiting plants of *Whitania aristata* were collected from the Santa Cruz Coast in a location

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Table 1
^{1}H and ^{13}C NMR (300 and 75 MHz, $CDCl_3$) spectroscopica data for Witharistatin.

Position	Witharistatin	
	δ 1H, multiplet (*J* in Hz)	δ ^{13}C JMOD
1		202.44 (C)
2	6.25 (d, 9.9)	132.95 (CH)
3	6.95 (d, 5.9, 9.9)	142.13 (CH)
4	3.80 (d, 5.9)	70.50 (CH)
5		62.84 (C)
6	3.20 (brs)	60.50 (CH)
7	1.37, 2.18	31.70 (CH_2), 31.70 (CH_2)
8		28.82 (CH)
9		45.11 (CH)
10		48.39 (C)
11		22.20 (CH_2)
12		33.30 (CH_2)
13		47.27 (C)
14		57.50 (CH)
15		34.82 (CH_2)
16	5.53 (brs)	125.13 (CH)
17		155.66 (C)
18	0.80 (s)	16.70 (CH_3)
19	1.45 (s)	17.69 (CH_3)
20	2.53	36.37 (CH)
21	1.12 (s)	17.25 (CH_3)
22	4.42 (m)[a]	79.44 (CH)
23	2.53	31.50 (CH_2)
	1.90	
24		153.05 (C)
25		126.13 (C)
26		167.17 (C)
27	4.36 (m)	57.96 (CH_2)
28	2.04 (s)	20.34 (CH_3)
OH	2.89 (brs)	

[a] Signal partially overlapped by that of H-27.

known as Taganana in Tenerife, Canary Islands (Martín-Herrera et al., 2007, 2008).

2.2. Extraction and isolation

The dry *Whitania aristata* material recollected (1.50 kg) was successively extracted with *n*-hexane (28.58 g), dichloromethane (28.30 g), ethyl acetate (FE fraction, 15.66 g), *n*-butanol (FB fraction, 22.76 g), methanol (FM fraction, 59.76 g) and finally water (FW fraction, 201.54 g). The aqueous diuretic active fraction (FW) was partitioned in the mixture hexane–dichloromethane–methanol (2:1:1, v/v/v), and the soluble fraction was separated by column chromatography on Sephadex LH-20, eluting with the same solvent mixture. The steroidal fractions (according to TLC dichloromethane: ethyl acetate, 5:45, v/v) were joined and then washed with chloroform to obtain 21.0 mg of withaferin A (**1**), as a white powder. From the steroidal residue, witharistatin (18.0 mg) was purified by preparative TLC using the same solvent system as above and recovered with methanol.

Witharistatin (**2**). Amorphous powder; $[\alpha]_D$: +103.4, ($CHCl_3$; c = 0.29); UV ($CHCl_3$) λ_{max} nm ($\log \varepsilon$): 325 (4.02), 235 nm (3.52); for ^{1}H NMR and ^{13}C NMR spectra see Table 1; HR-EI/MS: 468.2521 (calcd, for $C_{28}H_{36}O_6$, 468.2491); EI/MS: *m*/*z* (%): 468 M^+ (2.15); 453[M^+-CH_3] (6.8); 435 [$M^+-CH_3-H_2O$] (9.6); 417 [$M^+-CH_3-2H_2O$] (3.11); 141 [$C_7H_9O_3]^+$ (100%); 123 [$C_7H_9O_3]^+-H_2O$ (65%); 95 [$C_7H_9O_3]^+-H_2O-CO$ (36).

2.3. Animals

Male albino Sprague-Dawley rats (180–210 g) and male and female albino Swiss mice (20–24 g) obtained from the Central Animal House, University of La Laguna, were used for the experiments, according with the guidelines of the European Community Council Directive 86/609, for the handling and use of laboratory animals.

2.4. Drugs

Hydrochlorothiazide (HCT; Sigma Chemical Co.) was used as a diuretic reference drug.

2.5. Diuretic activity

Diuretic activity was determined following the methods of Kau et al. (1984) with modifications (Martín-Herrera et al., 2007, 2008). Four groups of rats were orally administered 5 ml/kg bw of ethyl acetate, *n*-butanol, methanol and water fractions of *Whitania aristata*, at 100 mg/kg, and one group of rats was orally administered 5 ml/kg bw of hydrochlorotiazide (HCT) at 10 mg/kg. Control rats received the same amount of deionized water (5 ml/kg bw). Urinary volume was recorded at 2 h intervals for 8 h. Cumulative urine excretion was calculated in relation to body weight and expressed as ml/100 g bw. Electrolyte (Na^+, K^+) concentrations were expressed as meq./100 g. pH, density and conductivity were estimated from a pooled urine sample of each pair of rats at the end of the experiment (8 h) (data not shown).

On the other hand, the isolated compounds, withaferin A and witharistatin, and a mixture of them at 5 and 10 mg/kg were also analyzed as diuretics, following the same method, but using four groups of 16 mice each, and the urine was collected at 2 h intervals for 6 h.

2.6. Analytical procedures

The melting point was determined on a Fisher-Johns apparatus. Optical rotations were measured in $CHCl_3$ at 25 °C on a PerkinElmer 341 polarimeter. NMR measurements were performed on a Bruker AMX300 in $CDCl_3$ solutions containing TMS as the internal standard at 300 MHz for ^{1}H and 75.4 MHz for ^{13}C. The high-resolution mass spectrum was measured by matrix assisted laser desorption ionisation (MALDI) mass spectrometry. Exact masses were measured by a Voyager DE mass spectrometer. Column chromatography was carried out on Merck silica gel 60 (230–400 mesh ASTM) and TLC on Merck silica gel 60 F_{254} plates.

For diuretic activity, Na^+ and K^+ concentrations were measured using a Jenway Corp. model PFP7 flame photometer. pH and conductivity (data not shown) were directly determined on fresh urine samples (Martín-Herrera et al., 2007, 2008).

2.7. Statistical analysis

Data are expressed as the means ± SEM for the number of animals in each group. The statistical evaluation was carried out by analysis of variance (ANOVA) and the difference between the means of treated groups and the non-treated control groups was evaluated by the Student's unpaired *t*-test. A probability level lower than 0.05 was considered as statistically significant. Sigma plot (version 8.0) software was used for statistics and plotting.

3. Results

The physico-chemical withanolides characteristics, and the different parameters analyzed for the different *Whitania aristata* extracts and the new isolated compounds in the test animals, are included in Fig. 1 and Tables 1–3.

3.1. Physico-chemical characterization of compounds

After solvent partition and chromatographical separation of the most diuretic and polar fraction obtained from *Whitania aristata*