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Diuretic activity of some *Withania aristata* Ait. fractions

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ABSTRACT

We previously reported on the significant dose-dependent diuretic effects produced in laboratory rats by hot water infusions and methanol extracts of *Withania aristata* Ait., where notable increases were observed in the excretion of water and sodium, with an interesting potassium-saving effect. The present study gives the results of the diuretic effects in rats of the hexane, dichloromethane, ethyl acetate, butanol and methanol–water fractions of the previously studied methanol extract.

Water excretion rate, pH, density, conductivity and content of Na^+, K^+ and Cl^- were measured in the urine of the rats when subjected to hypersaline conditions. Of the above fractions, the methanol:water extract (100 mg/kg) showed the most interesting diuretic activity (25%; $^{**}p < 0.01$), which suggested that increase in the polarity of the extracting solvent led to an increase in the concentration of the polar compounds responsible for the diuretic effect. These data, together with previous results on the aqueous and methanol extracts, reaffirm assertions made regarding the effectiveness of the extracts of this plant against urinary pathologies in the Canary Islands folk medicine.

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1. Introduction

Medicinal plants can be important sources of previously unknown chemical substances with potential therapeutic effects. The medicinal use of plants is an ancient tradition, far older than the contemporary sciences of medicine, pharmacology and chemistry. The World Health Organization has estimated that over 75% of the world's population still relies on plant-derived medicines, usually obtained from traditional healers, for its basic health-care needs (Farnsworth et al., 1985).

The flora of the Canary Islands is highly endemic, and the folk medicine based on endemic plants has a deeply rooted tradition. One of the endemic plants most commonly used is *Withania aristata* Ait. (Solanaceae), popularly known as "orobal" or "sáquido". Aerial portions and fruit of this species have long been used as a scarring agent, antispasmodic, for rheumatic and eyes problems, otitis, as well as for insomnia, constipation and urinary pathologies (Darias et al., 1989, 2001; Jaén, 1989; Pérez-Paz and Hernández, 1999).

Previous studies by our group have shown that hot water infusions and methanol extracts obtained from the leaves of flowers and immature fruit of this species produced dose-dependent diuretic activity in rats (Martín-Herrera et al., 2007).

On the other hand, partial studies on the chemical composition of *Withania aristata* have isolated phytosterols, oleoresins, withaminol (Valera and Santos, 2002) and withanolides (González et al., 1972, 1974). These compounds seems to be characteristic of the Solanaceae (Ganzera et al., 2003; Subbaraju et al., 2006; Damu et al., 2007; Pan et al., 2007). A large amount of experimental data clearly demonstrate that plants in the genus *Withania* can variously have anti-inflammatory, immunoprotective, anticancer, hepatoprotective, hypocholesteremic, antioxidant, antiartritic, anxiolytic, fungicidal, antibacterial, antimalarial and trypanocidal effects (Choudhary et al., 1995; Bhattacharya et al., 2000; Jayaprakasam and Nair, 2003; Owais et al., 2005; Abe et al., 2006; Malik et al., 2007; Muregi et al., 2007; Rasool and Varalakshmi, 2007; Saritha and Naidu, 2007; Saxena et al., 2007; Visavadiya and Narasimhacharya, 2007; Widodo et al., 2007).

The objective of the present study was to investigate the diuretic effectiveness of hexane, dichloromethane, ethyl acetate, *n*-butanol and methanol-aqueous fractions obtained from the methanol extract of *Withania aristata* in laboratory rats.

2. Materials and methods

2.1. Plant material

The leaves of flowering and immature fruiting plants of *Withania aristata* were collected from the Santa Cruz Coast in a location known as Taganana in Tenerife, Canary Islands (Spain) at 75 m alti-

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tude above sea level and labeled Ex. NE. UTM E381093-N3160004. They were identified by Dr. Pedro Pérez de Paz, Department of Plant Biology, University of La Laguna (Tenerife, Spain), where voucher specimens have been deposited (TFC 44199).

2.2. Extracts preparation

The leaves of flowering and immature fruiting *Withania aristata* were air-dried in an oven at 40 °C for 4 days and then the dry plant was cut and ground to a powder by mechanical milling.

The dried powdered plant material was submitted to continuous extraction in a Soxhlet extractor for 5 days using 100% methanol as a solvent. The solvent was then eliminated by vacuum distillation in a rotary vacuum evaporator (Buchler Corp.), representing a yield of 10.39% of the dry material extracted. The methanol residue obtained was partitioned between water and organic solvents of increasing polarities, to yield five new fractions, including: hexane (Fr. *n*-hexane), dichloromethane (Fr. CH_2Cl_2), ethyl acetate (Fr. EtOAc), *n*-butanol (Fr. *n*-BuOH) and methanol–H_2O (Fr. MeOH:H_2O), representing the water-soluble remaining extract. The yields obtained for each fraction with respect to the dry material were: Fr. *n*-hexane 10.2%, Fr. CH_2Cl_2 25%, Fr. EtOAc 10.7%, Fr. *n*-BuOH 3.9% and Fr. MeOH:H_2O 6.6%.

For pharmacological studies, the fractions were given orally to laboratory rats as a suspension in a 5% Tween 80 aqueous solution in a final volume of 5 ml/kg body weight (bw). In this paper, the doses employed are expressed as mg of the dried extract per kg bw.

2.3. Animals

Male albino Sprague–Dawley rats (180–210 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.

2.4. Drugs

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

2.5. Diuretic activity

Diuretic activity was determined following the methods of Kau et al. (1984), with minor modifications. Male rats were divided into seven groups, of eight animals each, in laboratory cages. They were fed laboratory diet *ad libitum* and allowed free access to drinking water. They were exposed to a 12-h light:12-h dark cycle at 22 °C. Eighteen hours before testing, the animals were fasted overnight, with free access to tap water only. Then all animals were given an oral loading of normal saline (5% bw). Subsequently, five groups of rats were orally administered 5 ml/kg bw of the fractions of *Withania aristata* at 100 mg/kg of weight, and one group of rats received orally 5 ml/kg bw p.o. of HCTZ at 10 mg/kg. Control rats received the same amount of deionised water (5 ml/kg bw). Immediately after administration, the rats were paired and placed in metabolism cages. Urine was collected in a graduated cylinder and its 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^+, Cl^-) concentrations, pH, density and conductivity were estimated from a pooled urine sample of each pair of rats at the end of the experiment (8 h) and expressed as mequiv./100 g bw.

2.6. Analytical procedures

Na^+ and K^+ concentrations were measured using a Jenway Corp. model PFP7 flame photometer. The instrument was calibrated with standard solutions containing different concentrations of Na^+ and K^+. Cl^- concentrations were determined by direct potentiometry, using an ion-selective chloride electrode (Orion 9417B) and an Ag/AgCl reference electrode with a double junction (Orion 90-02). The potentials were measured with an Orion Ionalyzer 901. KNO_3 2 M was used as a standard in all the determinations; pH and conductivity were directly determined on fresh urine samples using a HI-8424 Hanna Instruments pH-meter and a LF-320 WTF conductivity meter, respectively. Density estimation was made by weighing with a Mettler AE163 (±0.1 mg) analytical balance on urine volume measured with a Nichiryo micropipette.

2.7. Statistical analyses

Results are expressed as the mean values ± S.E. (standard error of mean) of four pairs of rats. The statistical evaluation was carried out by analysis of variance (ANOVA). The difference between the means of treated groups and the non-treated control groups was evaluated by the Student's unpaired *t*-test and 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 different parameters analyzed for the fractions of *Withania aristata* in the test animals, as well as the HCTZ and control groups, are included in Tables 1 and 2.

Table 1 shows the urinary volume (ml/100 g/8 h) and other parameters related to excretion such as the conductivity, pH and density, and in Table 2 the electrolyte (Na^+, K^+, Cl^-) content (mequiv./100 g/8 h) in the urine of animals treated with *Withania aristata* fractions, HCTZ and untreated control groups.

Table 1
Effects of oral administration of *Withania aristata* fractions and hydrochlorothiazide (HCTZ) on urinary volume excretion, conductivity, pH and density

Group	Dose (mg/kg p.o.)	*n*	Urine volume (ml/100 g/8 h)	Diuretic index	Conductivity (mS/cm)	pH	Density (g/ml)
Control	–	24	5.14 ± 0.20	1.00	12.80 ± 0.54	7.43 ± 0.18	0.99 ± 0.003
HCTZ	10	4	7.24 ± 0.36***	1.40	14.73 ± 0.43*	7.40 ± 0.32	0.99 ± 0.001
Fr. *n*-Hexane	100	4	5.70 ± 0.30	1.11	14.10 ± 0.95	7.23 ± 0.24	0.99 ± 0.001
Fr. CH_2Cl_2	100	4	5.90 ± 0.40	1.15	13.83 ± 0.99	6.87 ± 0.22	0.99 ± 0.001
Fr. EtOAc	100	4	6.14 ± 0.19*	1.19	13.77 ± 1.36	7.69 ± 0.15	0.99 ± 0.001
Fr. *n*-BuOH	100	4	6.10 ± 0.29*	1.19	14.43 ± 0.77	6.20 ± 0.15	0.99 ± 0.001
Fr. MeOH:H_2O	100	4	6.41 ± 0.75**	1.25	14.68 ± 1.14	8.14 ± 0.02*	0.96 ± 0.03

Each value represents the mean ± S.E.M. Diuretic index = volume problem group/volume control group. *n* = Number of pairs used in each group.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ compared with the control group (Student's unpaired *t*-test).

Table 2
Effect of oral administration of *Withania aristata* fractions and hydrochlorothiazide (HCTZ) on urinary electrolyte excretion

Group	Dose (mg/kg p.o.)	*n*	Na^+ (mequiv./100 g/8 h)	K^+ (mequiv./100 g/8 h)	Cl^- (mequiv./100 g/8 h)	Saluretic index			Na/K
						Na	K	Cl	
Control	–	24	0.42 ± 0.03	0.19 ± 0.02	0.58 ± 0.04	1.0	1.0	1.0	2.21
HCTZ	10	4	0.66 ± 0.05***	0.31 ± 0.01**	0.99 ± 0.06***	1.57	1.63	1.71	2.13
Fr. *n*-Hexane	100	4	0.55 ± 0.02*	0.24 ± 0.01	0.72 ± 0.02*	1.31	1.26	1.24	2.29
Fr. CH_2Cl_2	100	4	0.51 ± 0.02	0.20 ± 0.08	0.70 ± 0.03	1.21	1.05	1.21	2.55
Fr. EtOAc	100	4	0.47 ± 0.05	0.18 ± 0.02	0.61 ± 0.09	1.12	0.95	1.05	2.61
Fr. *n*-BuOH	100	4	0.45 ± 0.02	0.19 ± 0.01	0.69 ± 0.03	1.07	1.00	1.19	2.37
Fr. MeOH:H_2O	100	4	0.52 ± 0.04*	0.30 ± 0.01*	0.66 ± 0.13	1.24	1.58	1.14	1.73

Values are expressed as mean ± S.E.M. Saluretic index = mequiv. problem group/mequiv. control group. *n* = Number of pairs used in each group.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ compared with the control group (Student's unpaired *t*-test).

3.1. Urinary excretion volume

The results showed that the reference diuretic HCTZ induced excretion values for water of 40% and that all the different fractions of *Withania aristata* tested in the present study produced an increase in the urinary volume excretion, except the Fr. *n*-Hexane which produced only a small (11%) increase (Table 1).

The Fr. CH_2Cl_2 produced a slightly better increase in the urine excretion (15%) than the above-mentioned Fr. *n*-hexane, remaining well below the value produced by the reference diuretic drug when compared with the control group.

The Fr. EtOAc and Fr. *n*-BuOH produced a similar and significant increase ($^*p < 0.05$) in urinary excretion (19%) which was higher than that shown by the above, less polar fractions, beginning to indicate that the polarity of the solvent appears to have an influence on the diuretic effect of the extract.

Finally, the Fr. MeOH:H_2O produced a notable and highly significant increase in urinary excretion of 25% ($^{**}p < 0.01$) when compared with the control group. This fraction showed the highest and most significant diuretic effect compared with the other four fractions evaluated.

Thus, in summary, results obtained by the different fractions showed that there was a parallel increase in the volume of urinary excretion as the polarity of the solvents increased (Table 1), suggesting that urine excretion seems to be due to the presence in the most polar solvents of naturally active polar compounds.

3.2. Electrolyte excretion

Table 2 shows the ionic excretion obtained after oral administration of *Withania aristata* fractions and HCTZ.

It was observed that sodium excretion was between 7% and 31% above that of the control group, but lower than that shown by HCTZ (57%). The data showed a remarkable parallelism among sodium and urine excretion. Although, the Fr. *n*-hexane produced a significant increase in the excretion of sodium (31%), the water excretion in this case was low (11%), and lower than that shown by all the other fractions studied.

Nevertheless, it should be noted that only the Fr. MeOH:H_2O sodium excretion (24%), as well as being statistically significant ($^*p < 0.05$), was in agreement with the effect noted on urine excretion (25%).

The data for potassium excretion showed that only the Fr. MeOH:H_2O produced a significant increase (58%), which was very close to the value obtained using the HCTZ (63%) as a reference diuretic. This can also be noted from its appreciably low Na/K index (1.73), which was notably lower than that of HCTZ (2.13) (Table 2).

Finally, the ionic excretion obtained for all the tested fractions showed that, as the polarity of the solvents increased, the sodium and potassium excretion decreased, except for the Fr. MeOH:H_2O which diverged from this tendency and showed a significant increase in excretion for both of these electrolytes.

3.3. Other physicochemical parameters

Table 1 shows also the results obtained for the pH, density and specific conductivity of the urine after oral administration of the *Withania aristata* fractions. It can be seen that the pH increased significantly only with the Fr. MeOH:H_2O; perhaps this increase was due to an active substance present in this fraction which was capable of causing metabolic changes, and thus increasing the pH. The other fractions produced no observable changes in either pH or density.

The specific conductivity, which is an indirect measure of the ionic content of the urine, was increased by all the fractions when compared with the control. The Fr. *n*-hexane showed a considerable increase in conductivity, which appears to be due to the decrease in urinary volume excretion. It should be noted that conductivity depends on the concentration (equiv./l), and not on the total quantity (equiv.) of ions. However, the Fr. MeOH:H_2O also showed an interesting value for specific conductivity, probably related with its remarkable level of ionic excretion, which increased in comparison with the control group from 12.80 to 14.68 mS/cm, similar to the value produced by the HCTZ (14.73; $^*p < 0.05$). This finding was not obtained with any of the other fractions.

4. Discussion and conclusions

In a previous study carried out with infusions and a methanol extract of *Withania aristata*, we concluded that the diuretic action shown by the aqueous extract was not due to the high quantities of potassium found in the plant, since the methanol extract also produced diuretic activity. We noted that in contrast to the infusion in whose water preparation it occurs a removal of salts, with the methanol this salts removal did not generate, so we therefore strongly concluded that the diuresis produced by the methanol extract could not have been due to potassium content (Martín-Herrera et al., 2007). This fact reaffirms the idea that the diuretic activity of *Withania aristata* was not due to its content of potassium salts, and it is more reasonable to hypothesize a diuretic effect not related to an osmotic-type mechanism.

Present data obtained using the purified *Withania aristata* fractions further demonstrate the common diuretic effects demonstrated by the infusions and methanol extracts of this species. Thus, we have observed a tendency in the increase of water excretion with an increase in the polarity of the fractions obtained, achieving a maximum expression and statistical significance with the most polar fraction (Fr. MeOH:H_2O), that reaches the notable value of

25% diuretic activity when compared with the control group. With this fraction, we also found a noteworthy and significant increase in excretion of potassium, reaching a value of 58% in comparison with the control group, and similar to that observed with HCTZ (63%). Along with this datum, we noted a low value of 1.73 for the Na/K ratio.

In contrast with the previous assays carried out comparing aqueous and methanol extracts which showed an interesting K^+-saving effect at low and intermediate doses (Martín-Herrera et al., 2007), in the present study of the Fr. MeOH:H_2O the above K^+-saving effects were not observed. It is probable that at low dosages of the aqueous and methanol extracts of *Withania aristata*, the substances responsible for the powerful K^+-saving effect were not found in sufficient concentrations as occurred with the purified fraction. This seems to be due to the existence, at least of two different mechanisms, one of which produces notable diuresis with a sparing of potassium and another with very strong diuresis in which there is a clear tendency to lose the potassium-conservative effect.

The active principle/s responsible for the diuretic effects of the Fr. MeOH:H_2O of this species is/are, so far, not known, but preliminary phytochemical analysis carried out with the methanol extract and with the Fr. MeOH:H_2O revealed the presence of polar compounds such as flavonoids and steroidal lactones. As some previous investigations on the composition of *Withania aristata* have suggested the presence in the plant of steroidal lactones such as the withanolides (González et al., 1972, 1974), one can suppose that these substances might be responsible, at least in part, for the observed diuretic activity and that they may act individually or synergistically. Previous studies have demonstrated also that there are several compounds which could be responsible for the plants diuretic effects such as flavonoids, saponins or organic acids (Maghrani et al., 2005). The effect may be produced by stimulating regional blood flow or initial vasodilation (Stanić and Samaržija, 1993), or by producing inhibition of tubular reabsorption of water and anions (Pantoja et al., 1991), with the result in both cases being diuresis.

In summary, revisiting all the data presently available on the *Withania aristata* fractions, there has been a very interesting saluretic diuretic effect noted, which is not due to an osmotic mechanism resulting from the presence of potassium salts in the plant. It was also noted that the diuretic effect of *Withania aristata* was different than that produced by HCTZ, since at moderate dosages there was a beneficial potassium retention effect.

Finally, our data seems to indicate that this diuretic effect is associated with the presence in the plant of active principles of a polar nature, where the withanolides may be the main chemical protagonists of this activity.

The present results provide further support to explain the traditional folk-medicine use of *Withania aristata* as a diuretic agent by the Canary Island population. Additionally, this species may be of use in treatment of bacterial urinary infections through the action of "therapeutic lavage" (when taken with a sufficient quantity of liquid), based on its content of the withanolides, which may exhibit a certain degree of antibacterial activity (Arora et al., 2004).

Further research is under way in our laboratory to elucidate the mechanism of this diuretic action, and particularly the role of its active components such as the withanolides.

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