ORIGINAL ARTICLE

DIURETIC AND ANTI-INFLAMMATORY EFFECTS OF A CRUDE STEROIDAL SAPONIN FROM THE RHIZOMES OF *RUSCUS ACULEATUS* L. IN RATS

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Abstract

The present study was designed to evaluate the diuretic and anti-inflammatory effects of a crude steroidal saponin from the rhizomes of *Ruscus aculeatus* L. (*Asparagaceae*). In order to evaluate the diuretic effect, the saponin was administered orally in different doses to saline loaded Charles River Wistar (Crl:WI) rats, the urinary volumetric excretion and the urinary excretion of key electrolytes being recorded at several time intervals. For the assessment of the anti-inflammatory effect, the acute phase bone marrow response, the phagocytic capacity and the total antioxidant response were evaluated. The results of this study demonstrated significant and dose-dependent diuretic and anti-inflammatory effects for the crude steroidal saponin extracted from the rhizomes of *Ruscus aculeatus* L., which could lead to the development of some pharmaceutical products with natural compounds potentially active in disorders of the urinary tract.

Rezumat

Studiul a avut ca obiective evaluarea efectelor diuretice şi antiinflamatoare ale saponinei steroidice brute extrasă din rizomii speciei *Ruscus aculeatus* L. (*Asparagaceae*). Pentru a evalua efectul diuretic, saponina a fost administrată pe cale orală, în doze diferite, unor şobolani Crl:WI hidrataţi, cu evaluarea excreţiei volumetrice urinare şi a excreţiei unor electroliţi la diferite intervale de timp. Pentru determinarea efectului antiinflamator, s-au evaluat răspunsul medular de fază acută, indicele fagocitar şi capacitatea antioxidantă totală. Rezultatele au demonstrat efecte semnificative statistic şi doză-dependente pentru saponina steroidică brută extrasă din rizomii speciei *Ruscus aculeatus* L. ceea ce ar putea duce la dezvoltarea unor produse cu compuşi naturali potenţial activi în afecţiuni ale tractului urinar.

Keywords: *Ruscus aculeatus* L., steroidal saponin, diuretic, anti-inflammatory

Introduction

Ruscus aculeatus L., Butcher's broom (*Asparagaceae* family, formerly *Ruscaceae)*, is a small evergreen shrub native to Mediterranean, Southern and Western Europe [9]. *Ruscus aculeatus* L. was traditionally regarded in Western herbal medicine as a diuretic, anti-inflammatory and phlebotherapeutic agent and was used to treat a variety of conditions including swelling and heavy legs, being used also locally in arthritis [15]. More recently, after the discovery of the venotonic properties of the two main active principles, the steroidal aglycons ruscogenin and neoruscogenin, *Ruscus aculeatus* L. has been used as an effective therapy for symptoms associated with varicose veins and haemorrhoids [3]. Also, the vasoconstrictive properties of the plant recommended its use in the treatment of orthostatic hypotension [11].

Despite the large number of published studies concerning the therapeutic potential of *Ruscus aculeatus* L., very few reports investigated the diuretic and anti-inflammatory effects claimed in folk medicine, so far. Thus, our study was designed to evaluate *in vivo* the potential diuretic and anti-inflammatory effect of a crude steroidal saponin extracted from the rhizomes of *Ruscus aculeatus* L. in Crl:WI rats.

Materials and Methods

Plant material

The underground parts (rhizomes) of *Ruscus aculeatus* L. were harvested from Lipova Hills, Arad County, Romania, in October 2014. After identification, a voucher specimen of the plant was deposited in the Herbarium of the Department of Pharmaceutical

Botany, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania (item no. 12a).

Preparation and chemical analysis of the crude steroidal saponin

After a drying period, the vegetal product was grinded to a fine powder. For the isolation of the crude steroidal saponin, the powder (50 g) was defatted with ethylic ether in the Soxhlet apparatus, for six hours, and then extracted two times with 500 and 250 mL methanol. The resulted methanolic solution was concentrated under reduced pressure in a rotavapor Hannvapor HS-2005V, and further precipitated in ethylic ether, generating the crude steroidal saponin. The precipitate (steroidal saponin) was filtrated in a Büchner funnel under vacuum and then desiccated with anhydrous calcium chloride, for 3 days, until complete drying [2]. The identification and quantification of ruscogenin and neoruscogenin, the main active principles from the crude saponin, was performed by a HPLC-MS/MS technique, using ruscogenin and neoruscogenin (Planta Analytica) as external standards [16]. The two active constituents were determined after the hydrolysis with sulphuric acid in isopropanol 70% [4]. Chromatographic separation was performed on a reverse phase column (Zorbax SB-C18, 100 × 3.0 mm i.d., 3.5 µm particle), under isocratic conditions using a mobile phase of 70/30 (v/v) acetonitrile/0.1% (v/v) formic acid in water containing 10 µM sodium acetate. The analysis was performed with an 1100 Series HPLC system (Agilent Technologies, USA) coupled to an Agilent mass spectrometer. Chromatographic and mass spectrometric data acquisition was performed using ChemStation software (Agilent) [16].

Animals

Adult male Charles River Wistar (Crl:WI) rats with a medium weight of 183 ± 6 g were obtained from the Practical Skills and Experimental Medicine Centre of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca (Romania). The rats were housed in polycarbonate type IV-S open-top cages (Tecniplast, Italy) and maintained under standard conditions (22 ± 2°C, a relative humidity of 45 ± 10%, 12:12 h light:dark cycle). The animals had access to a standard pelleted food (Cantacuzino Institute, Bucharest, Romania) and filtered water *ad libitum* throughout the experiment, except for the day when the test substances were administered. All experimental protocols were approved by the Ethics Committee of the University and were conducted in accordance with the EU Directive 86/609/EEC, which regulates the use of laboratory animals for scientific research.

Diuretic effect

For the evaluation of the diuretic activity of the crude steroidal saponin from the rhizomes of *Ruscus aculeatus*, a method using isotonic saline solution as hydrating fluid was used [7]. Five groups of rats (n = 6) were used. The negative control group of rats was treated by intragastric route with 25 mL/kg isotonic saline solution while the positive control group was treated by the same route with 10 mg/kg furosemide, a reference diuretic drug, dissolved also in a volume of 25 mL/kg isotonic saline solution. The other three groups of rats were treated also by intragastric route with 125, 250 and 500 mg/kg b.w. steroidal saponin from *Ruscus aculeatus*, suspended in the same volume of 25 mL/kg isotonic saline solution.

Afterwards, the animals were placed individually in metabolic cages, the environmental temperature being maintained at 24ºC. The cumulative urine output was recorded for each animal at 1 h, 3 h, 5 h and 24 h after the administration of a single dose from the tested substances [13]. The urinary volumetric excretion (UVE) was calculated as follows:

$$UVE = (Vol.\ collected/Vol.\ administered) \times 100.$$

Also, the urinary excretion of sodium, potassium and chloride ions ($U_{Na}V$, U_KV and $U_{Cl}V$) was determined twice in the collected urine samples, at 5 h and 24 h after the substance administration, by a potentiometric method, using a Vitros-250 Chemistry System auto-analyser (Johnson and Johnson Clinical Diagnostic), being expressed in mEq/kg.

Anti-inflammatory effect

In order to evaluate the anti-inflammatory effect of the crude steroidal saponin from the rhizomes of *Ruscus aculeatus*, the acute phase bone marrow response, the phagocytic capacity and the total antioxidant response (TAR) were determined in Crl:WI rats [1, 10]. The animals were divided in four groups (n = 6). Group 1 (inflammation) was treated i.m. with turpentine oil, a pro-inflammatory agent (0.6 mL/100 g b.w.). Group 2 (diclofenac) received the same pro-inflammatory treatment as Group 1, but in addition was treated intraperitoneally with a reference anti-inflammatory drug, diclofenac (50 mg/kg b.w.). Group 3 (saponin 40 mg/kg) was treated with the pro-inflammatory substance and the steroidal saponin administered intraperitoneally (40 mg/kg b.w.). Group 4 (saponin 400 mg/kg) was treated with the pro-inflammatory substance and the steroidal saponin administered intraperitoneally in a higher dose (400 mg/kg b.w.).

After 24 h, all animals were anesthetized with ketamine (90 mg/kg b.w.), blood samples being collected by retro-orbital sinus puncture. Samples used for the determination of the acute phase bone marrow response and the phagocytic capacity were collected in EDTA-coated recipients, while samples used for the evaluation of TAR were collected without anticoagulant. Serum separation was performed by centrifugation (1500 g; 10 min.), all samples being tested immediately after collection.

The acute phase bone marrow response was assessed by the determination of the total leukocyte count and leukocyte profile expressed as percentage. The phagocytosis test was performed by incubating at 37ºC for 30 min a blood sample from the tested animals with an *Escherichia coli* suspension (4×10^6 germs/mL in normal saline). The mixture ratio was 0.2 mL blood sample/20 µL *E. coli* suspension. The count was performed with an Olympus microscope on May-Grunewald-Giemsa stained smears, prepared from the incubation mixture [6]. The phagocytic index was calculated as the percentage of leukocytes that phagocyted at least one germ. The total anti-oxidant response (TAR) was determined in the serum of the treated animals by a colorimetric method [5]. Briefly, the hydroxyl radical was produced by the Fenton reaction and reacted with a colourless substrate (o-dianisidine) to produce the dianisyl radical, with a yellow-brown colour. The assay was calibrated with Trolox, the results being expressed as mmol Trolox Equiv/L.

Statistical analysis

Data were expressed as mean values ± SEM and were statistically analysed by one way ANOVA method. The differences between the treated groups and the negative control group were evaluated by Dunnett's 't' test, p values < 0.05 being considered statistically significant.

Results and Discussion

Phytochemical analysis

The active constituents of *Ruscus aculeatus* L. rhizomes are considered to be steroidal saponins. The yield of crude saponin extraction was 15.5%. The HPLC-MS/MS assay showed that ruscogenin and neoruscogenin were the main active principles from the crude saponin extracted from the rhizomes, their content being 0.329% and 0.8239%, respectively.

Diuretic effect

As shown in Figure 1, the steroidal saponin from the rhizomes of *Ruscus aculeatus* L. produced a dose-dependent gradual increase in the urinary volumetric excretion (UVE) only from the third hour after the oral administration, the effect being more intense and statistically significant at 5 h and 24 h. The reference loop diuretic, furosemide, augmented the urinary volumetric excretion from the first hour after the oral administration, the effect increasing sharply at 3 h, then reaching a plateau before decreasing, as expected for a high ceiling diuretic drug. 24 h after the oral administration, the 500 mg/kg dose of crude steroidal saponin from the rhizomes of *Ruscus aculeatus* L. produced a urinary volumetric excretion (UVE) of 78.37, superior to the UVE of furosemide (58.22).

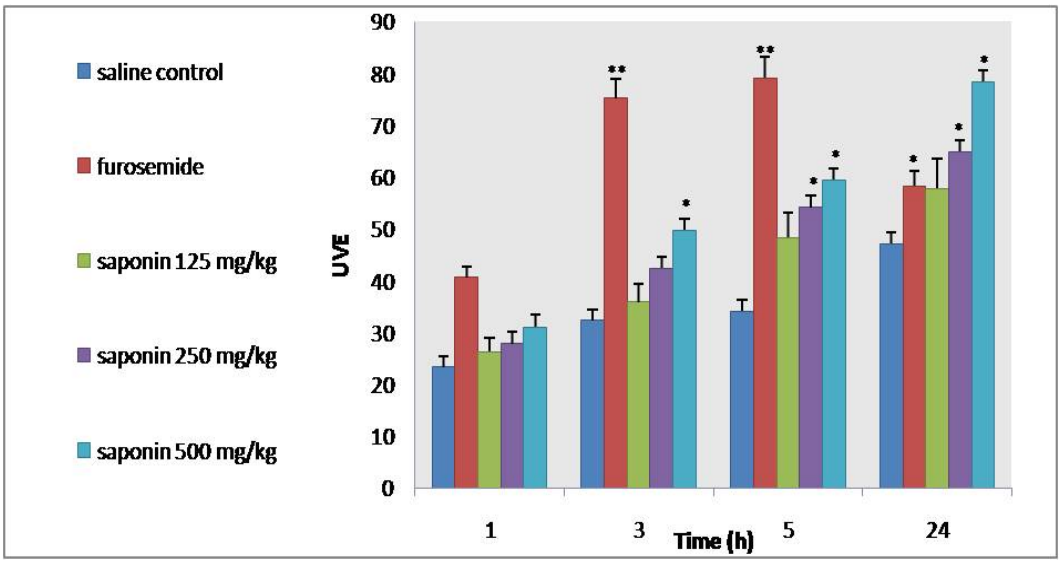

Figure 1.

Effect of the crude steroidal saponin on urinary volumetric excretion (UVE) recorded at 1, 3, 5 and 24 h in Crl:WI rats (* $p < 0.05$ *vs.* saline control and ** $p < 0.01$ *vs.* saline control)

Also, the crude steroidal saponin from *Ruscus aculeatus* L. increased the urinary excretion of Na^+, K^+ and Cl^- ions ($U_{Na}V$, U_KV and $U_{Cl}V$).

As shown in Table I, the urinary excretion of the aforementioned electrolytes produced by the crude steroidal saponin, was superior to the negative control group, and presented a similar pattern with the diuretic effect, being more intense in the 5 h - 24 h time interval. The most significant excretion of the tested electrolytes was produced by the 500 mg/kg dose of crude steroidal saponin with $U_{Na}V$ and U_KV values of 5.33 and 4.11 mEq/24 h/kg, 24 h after the substance administration.

Table I

Effect of the crude steroidal saponin on urinary excretion of sodium ($U_{Na}V$), potassium (U_KV) and chloride ($U_{Cl}V$) in saline loaded Crl:WI rats, 5 h and 24 h after the substance administration

Group (Dose)	$U_{Na}V$ (mEq/5 h/kg)	U_KV (mEq/5 h/kg)	$U_{Cl}V$ (mEq/5 h/kg)	$U_{Na}V$ (mEq/24 h/kg)	U_KV (mEq/24 h/kg)	$U_{Cl}V$ (mEq/24 h/kg)
Saline control (25 mL/kg)	1.75 ± 0.12	1.04 ± 0.10	2.74 ± 0.14	2.21 ± 0.48	1.78 ± 0.29	4.04 ± 0.33
Furosemide (10 mg/kg)	7.59 ± 0.63**	4.91 ± 0.26*	13.25 ± 0.97*	8.32 ± 0.85**	5.71 ± 0.49**	15.45 ± 1.17*
Saponin (125 mg/kg)	1.89 ± 0.21	1.63 ± 0.20*	3.83 ± 0.42	3.07 ± 0.38*	4.36 ± 0.87*	8.06 ± 0.76*
Saponin (250 mg/kg)	2.17 ± 0.15*	1.59 ± 0.17*	3.93 ± 0.25*	3.58 ± 0.44*	3.43 ± 0.51*	6.98 ± 0.69*
Saponin (500 mg/kg)	4.84 ± 0.19*	3.42 ± 0.37*	9.44 ± 0.87*	5.33 ± 0.61*	4.11 ± 0.49*	10.24 ± 0.73*

Note: * $p < 0.05$ *vs.* saline control and ** $p < 0.01$ *vs.* saline control; values are expressed as mean ± SD

The results of the experiment showed a moderate but prolonged diuretic activity of the crude steroidal saponin from the rhizomes of *Ruscus aculeatus* L, contrasting with the intense and brief effect of furosemide, the reference diuretic drug.

The diuretic and saluretic effect of the crude steroidal saponin from *Ruscus aculeatus* L. although inferior to furosemide, was statistically significant at 5 h and 24 h after the oral administration, and could be at least partially attributed to a tubular effect with a decrease in water and electrolytes reabsorption.

Anti-inflammatory effect

The results of the assessment of the anti-inflammatory effect are presented in Table II.

Table II

Effect of the crude steroidal saponin on the acute phase bone marrow response, phagocytic capacity and total antioxidant response (TAR)

Group	Leukocytes no/mm^3	Neutrophils %	Monocytes %	Lymphocytes %	Phagocytic index	TAR
Inflammation	13654 ± 1600.77	70.2 ± 4.49	8 ± 1.58	21.8 ± 2.94	48.8 ± 6.72	0.226 ± 0.02
Diclofenac 50 mg/kg	6095 ± 1580.88*	60.4 ± 4.97*	7.2 ± 3.89	32.4 ± 4.33	22.4 ± 4.56*	0.202 ± 0.04
Saponin 40 mg/kg	5690 ± 913.37*	65.2 ± 4.60	4.4 ± 1.54*	30.4 ± 1.67	14.4 ± 1.67*	0.67 ± 0.06*
Saponin 400 mg/kg	4970 ± 709.40*	64 ± 3.74*	2 ± 1.41*	14.8 ± 3.63*	19.6 ± 2.60*	0.58 ± 0.04*

Note: Asterisk indicate significant differences at levels of * $p < 0.05$ *vs.* saline control; Values are expressed as mean ± SD

The oral administration of the crude steroidal saponin extracted from the rhizomes of *Ruscus aculeatus* L. decreased the total leukocyte count and the percentage of neutrophils, indicating a reduction of the cellular component of the inflammation. Also, the crude steroidal saponin reduced the phagocytic index, the results being superior to the reference anti-inflammatory drug, diclofenac. Additionally, the administration of the saponin increased the total antioxidant response (TAR) in treated animals, with a possible decrease of reactive oxygen species, molecules involved in the induction of inflammatory processes.

The cellular events of an inflammatory response are typically characterized by increased leukocyte infiltration and accumulation of neutrophils [8]. Furthermore, an increased phagocytic capacity can cause injury and tissue damage in certain situations. Also, an increased oxidative stress can aggravate the inflammatory process by activation of particular transcription factors (NF-kB) or by an increased expression of the genes of some pro-inflammatory cytokines [12].

Thus, by decreasing the leukocyte number and the percentage of neutrophils and by increasing the antioxidant response, the crude steroidal saponin from *Ruscus aculeatus* L. showed a significant anti-inflammatory effect at cellular level.

Conclusions

The results of this study showed that the crude steroidal saponin from the rhizomes of *Ruscus aculeatus* L. (*Asparagaceae*) produced a significant and dose-dependent diuretic effect associated with an increase in urinary excretion of electrolytes. Also, the tested saponin produced a significant anti-inflammatory effect by cellular mechanisms. This species can be used as a source of natural compounds with potential uses in disorders of the urinary tract.

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