Original Article

Antileishmanial Activity of New Steroidal Saponin Isolated from the Flowers of *Allium Austroiranicum*

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Abstract

Background & Objective: Plants are reservoirs of bioactive compounds, which are known to be chemically balanced, effective and least injurious as compared to synthetic medicines. The current resistance and the toxic effects of the available drugs have brought the trend to assess the antileishmanial effect of various plant extracts and their purified compound/s. *Alliums* are rich sources of steroidal saponins, flavonoids, and sulphuric compounds of which steroidal saponins have recently received more attention due to their important pharmacological activities. *Allium austroiranicum* is a common edible vegetable in western regions of Iran, especially in "Chaharmahal and Bakhtiari" province, where it is named "Lopo" and is considerably used as a raw vegetable, flavoring agent, and as a medicinal plant.
Materials & Methods: The chloroform-methanolic extract was fractionated using MPLC, and the appropriate fractions were then subjected to isolation and purification of the constituents by HPLC. Structure elucidation was done using comprehensive spectroscopic methods including 1D and 2D NMR. Antileishmanial effects of the isolated compound against the promastigotes of *Leishmania major* were evaluated using MTT method.
Results: Phytochemical investigation of chloroform-methanol extract of the plant resulted in the isolation and identification of a Nicotianoside C related steroidal saponin and its chemical structure was determined as (25S)-5α-Spirostan-1β,3β-diol-3-O-{α-L-rhamnopyranosyl-(1→2)-{α-L-rhamnopyranosyl-(1→4)}-[β-D-glucopyranoside}. Investigation of in vitro antileishmanial activity of the isolated compound, in 10 and 50 and 100 µg/mL concentrations, exhibited significant leishmanicidal effects against the promastigotes of *Leishmania major*.
Conclusion: The results established a valuable basis for further studies about *A. austroiranicum* and anti-parasitic activity of steroidal saponins.

Keywords: *Allium austroiranicum*, Antileishmania, Saponin, Structure elucidation

Introduction

Today, a large number of medicinal compounds in the pharmaceutical market are obtained directly or indirectly from plants (1). As a valuable group of medicinal plants, *Allium* species belong to the Amaryllidaceae family and include about 900 species and 15 subspecies. These plants have been used by humans for centuries due to their remarkable aroma, flavor and medicinal properties (2). Plants of this genus are usually herbaceous, with different underground storage structures such as rhizomes, storage roots and specific onions, and have mainly distributed through the northern hemisphere (3). *Allium* species are widely used plants that are consumed worldwide, both as raw vegetables to make different dishes and as medicinal plants (4). *Allium* plants are used to treat many diseases especially hypercholesterolemia and

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hypertension (5, 6). While the bioactive compounds in these plants reduce the risk of cardiovascular diseases and affect the blood glucose level, they have also positive effects on the treatment of arthritis, toothache, chronic cough, constipation, parasitic infections, gynecological and infectious diseases (7). Antioxidant, antimicrobial, antiviral, antifungal, anti-thrombotic, anti-inflammatory, anti-spasm and anti-tumor effects are other biological activities explained for *Alliums* (8).

Phytochemically, *Allium* species are rich sources of important secondary metabolites including steroidal saponins, flavonoids, and organosulfur compounds. They also contain carbohydrates, proteins, vitamins and alkaloids (4).

Among these compounds, flavonoids have antioxidant effects by scavenging free radicals and inhibiting lipid peroxidase (9), Organosulfur compounds have cytotoxic effects and prevent tumor progression (10). Carbohydrates may interact with immune cells to modulate immune responses in the body (11), and saponins have antifungal, antitumor, cytotoxic, antispasmodic, cholesterol-lowering activities (1,12), anti-inflammatory, antimicrobial, antiviral, antiparasitic, antithrombotic, ion channel blocker and immune-modulating effects (13).

Due to their important therapeutic activities, saponins have recently received more attention. Saponins are a group of naturally occurring plant glycosides, characterized by their foam-forming properties in an aqueous solution (14). These compounds also lysis red blood cells through non-specific reactions with the membrane proteins (15).

Saponins are mainly found in roots, tubers, leaves, flowers or seeds (14). These compounds are structurally divided into two groups, triterpene saponins and steroidal saponins (16). While triterpene saponins are found in large amounts in dicotyledonous plants, the steroidal saponins are less abundant and are usually found in monocotyledonous plants (17).

Allium Austroiranicum with the local name "Lopo", is one of the endemic plants of the genus *Allium* in Iran. This plant is found on the rocky slopes of Zagros region and also to some extent in Isfahan, Yazd and Fars provinces. *A. austroiranicum* has pink to purple star-shaped flowers and is used as a fragrant vegetable and edible spice in the growing areas, especially in the Borujen region (18). The current paper reports the identification of a new steroidal saponin from the flowers of *A. austroiranicum* and *in vitro* evaluation of its leishmanicidal activity against the promastigotes of *Leishmania major.*

Materials & Methods

General experimental procedures

Medium pressure liquid chromatography (MPLC) was performed by a Buchi Gradient System C-605 apparatus using glass columns of LiChroprep® RP-18 (25-40 µm, Merck, Germany) and C-660 Buchi fraction collector. Thin-layer chromatography (TLC) performed on SiO_2 plates (Merck, Germany) with BuOH: CH_3COOH: H_2O (4:1:5 v/v/v) (BAW) as a mobile phase and cerium sulfate in 2N H_2SO_4 and natural product (NP) as reagents for visualizing the spots. All used materials were of analytical grade (Merck, Germany).

High-pressure liquid chromatography (HPLC) was performed by Waters 515 apparatus equipped with a refractive index detector (Waters 2414) and UV detector (Waters 2487), using semipreparative C18 column (Novapak® 7.8 × 300 mm, Waters, USA) in isocratic mode.

Nuclear magnetic resonance (H- and C-NMR) spectra were recorded by Bruker 400 MHz (H at 400 MHz, C at 100 MHz) spectrometer, using solvent signal for calibration (CD_3OD: δH = 3.31, δC = 49.0). Distortionless enhancement by polarization transfer (DEPT) experiments was used to determine the multiplicities of C-NMR resonances. 2D heteronuclear single-quantum coherence (HSQC), interpulse delay set for ^{1}JCH of 130 Hz. Electrospray ionization mass spectroscopy (ESIMS) spectra were prepared by Waters QuattroII liquid chromatography-tandem mass spectrometry (LC/MS/MS), using methanol as the solvent.

Plant material

The whole plant of *A. austroiranicum* was collected from Borujen city (Chaharmahal and Bakhtiari, Iran), in the spring of 2019 and identified by a botanist. A voucher specimen (No. 3999) was deposited at the Herbarium of Department of Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Extraction and isolation

Flowers of *A. austroiranicum* were separated, air-dried in the shade, and powdered using a mill. The powder (254 g) was extracted at room temperature in a four-step extraction method with increasing solvent polarity using the solvents; hexane, chloroform, chloroform-methanol (9:1), and methanol. Extraction was done using the maceration method, performing each step three times with 1/5 L of solvent under occasional stirring.

The chloroform-methanol (9:1) extract of the sample was concentrated under vacuum, yielding a crude dried extract (9 g) which was fractionated by MPLC on an RP-18 column (36×460 mm) using a linear gradient solvent system of H_2O to CH_3OH. Fractions were analyzed by TLC (SiO_2, BAW 4:1:5 v/v/v) and similar fractions were mixed. Based on TLC and preliminary NMR analysis, the 10th fraction was considered richer in steroidal saponins, which was concentrated by a rotary evaporator and subjected to HPLC for further purification. The final purification of the fraction was performed by HPLC using a semi preparative C18 column (Novapak® 7.8 × 300 mm) and H_2O:CH_3OH (30:70) mobile phase in isocratic mode, resulted in the compound (1) (5 mg).

Evaluation of antileishmanial activity

Leishmania parasites

Cryopreserved *Leishmania major (L. major)* (MRHO/IR75/ER) were obtained from Department of Parasitology and Mycology, Isfahan University of Medical Sciences, Isfahan, Iran and were transferred to modified Nicole Novy Neal (N.N.N.) medium supplemented with streptomycin (100 µg/mL), and penicillin (100 U/mL). The promastigotes were then passaged in complemented RPMI 1640 with fetal calf serum (10% v/v), penicillin (100 U/mL), and streptomycin (100 µg/mL) and incubated at 25 °C. Antileishmanial activity was evaluated using promastigotes in logarithmic phase.

Antileishmanial assay

The antileishmanial assay was performed as described by Eskandarian, *et al* (19). Briefly, *L. major* promastigotes 4 × 10^5 in 400 µL complemented RPMI were cultured in wells of a 24-well plate. The steroidal saponin was dissolved in RPMI 1640 with the aid of 2% DMSO as co-solvent and added to the wells to make the final concentrations of 10 , 50 and 100 µg/mL. The plates were incubated at 25 °C for 2 days and the number of viable parasites was counted during the periods of 24, and 48 h. Amphotericin B at concentrations of 100 µg/mL and RPMI medium were used as positive and negative control, respectively. Plates were incubated at 24 ± 1 °C and the percentage of live promastigotes in each well was determined by the MTT assay at 24, 48 and 72 h. The promastigote viability was calculated by using the following equation: %viability= ((treated cells absorbance - blank absorbance)/ (negative control absorbance - blank absorbance)) ×100. Briefly, to perform MTT assay, 10 µL of 5% MTT solution was added to each well and was incubated for 4 h at 37 °C and 5% CO_2 in a dark condition. Then, 100 µL of 10% SDS solution (in 0.01 N hydrochloric acid) was added to each well and incubated again for 24 h under the mentioned condition.

Statistical analysis

Antileishmanial activities were reported as mean ± standard deviation (SD) and statically analyzed by one-way ANOVA and Tukey-Kramer test (SPSS V. 26). The significance level was considered at $P < 0.05$.

Results

A saponins-riched fraction of chloroform-methanol extract of *A. austroiranicum* was selected for final purification, resulting in the isolation and identification of a new steroidal

saponin (1) which was structurally related to the known steroidal saponin, Nicotianoside C. The chemical structure of isolated compound was determined using comprehensive spectroscopic methods and also by comparison of the spectral data with those reported in the literature. The isolated steroidal saponin exhibited a time dependent leishmanicidal activity against the promastigotes of *Leishmania major.*

Characterization of compound (1)

The steroidal saponin nature of compound (1) was confirmed by H and C-NMR spectra, including those related to steroidal part including a bundle of overlapped signals at δH 1.05 to 2.2 ppm and the existence of diagnostic and characteristic signals of saponins especially two tertiary methyls (3H, singlets: δH 0.81, δH 0.95; CNMR: δc 14.87, δc 17.52), two secondary methyls (3H, doublets: δH 0.71 (d, J=6.4), δH 0.86 (d, J=6.9); CNMR: δc 16.99, δc 16.21), three anomeric protons (δH 4.44, 4.55, 4.87) and related anomeric carbon signals (δc 104.28, 104.73, 104.96) (Table 1). Other diagnostic resonances in CNMR spectrum of compound (1) were δC 110.59 which was attributed to the C-22, δC 78.52 for C1 (C-OH), δC 78.30 for C3 (C-OH), δC 16.41 for $C6^{II}$ (Rha^{II}; CH_3) and δC 17.93 for C6III (RhaIII; CH3). In the ESIMS spectra, compound (1) showed a pseudomolecular ion peak at m/z 909.49 $[M+Na]^+$ in the positive-ion mode which together with the C-NMR data, suggested its molecular formula as $C_{45}H_{74}O_{17}$ (Table 1). Using the mass spectrometry (ESIMS) and NMR spectral data and comparing them with those reported in the literature (20, 22), the nature of the aglycon part of the compound was determined as smillagenin, which was finally confirmed by HSQC correlations.

Table 1. H-NMR and C-NMR data of aglycon part of compound (1)

Position	δH (int., mult., J in Hz)	δC (mult.)
1	3.27	78.52(CH)
2	1.33,1.52	30.79(CH_2)
3	3.48	78.30(CH)
4	2.24, 2.26	36.80(CH_2)
5	1.82	43.46(CH)
6	1.37	28.51(CH_2)
7	1.85, 1.89	32.41(CH_2)
8	1.52	33.16(CH)
9	0.86	57.28(CH)
10	-	37.09(C)
11	1.43 (1H, dd, 10.5, 2.5), 1.29 (1H, m)	22.01(CH_2)
12	1.64, 1.08	41.01(CH_2)
13	-	41.80(C)
14	1.05 (1H, m)	57.42(CH)

15	1.17, 1.97	32.70(CH_2)
16	4.30 (1H, q, 7.2)	82.19(CH)
17	1.64	62.70(CH)
18	0.81 (3H,s)	14.87(CH_3)
19	0.95 (3H,s)	17.52(CH_3)
20	1.80	42.92(CH)
21	0.86 (3H, d, 6.9)	16.21(CH_3)
22	-	110.59(C)
23	1.57, 1.48	34.99(CH_2)
24	1.48, 1.24	29.88(CH_2)
25	1.20	31.45(CH)
26	3.37, 3.22	67.85(CH_2)
27	0.71(3H, d, 6.4)	16.99(CH_3)

(400 MHz, 100 MHz; CD_3OD)

Determining the glycon part of compound (1), using the first anomeric proton position ($H1^I$; δH 4.44) and HSQC, H and C-NMR spectral data, especially the characteristic large coupling constant of $H1^I$ (1H, d, J= 7.6), the first sugar was determined as β-D-glucopyranoside. Completing the sugar chain structure elucidation, the observation of two characteristic anomeric proton broad singlets (δH 4.55 and 4.87) and two methyl groups observed as two overlapped doublets (δH 1.15 and 1.23; δC 16.41 and 17.93), determined the nature of two remaining sugars as α–L-rhamnopyranosides which were suggested to be attached to $C2^I$ and $C4^I$ of β-D-glucopyranoside, respectively. This was confirmed by the glycosylation shifts of $C2^I$ and $C4^I$ and also by the fragmentation peaks in the ESIMS spectrum due to the loss of sugar unites from the pseudomolecular ion (Table 2). According to the spectroscopic data, the chemical structure of compound (1) was finally determined as (25S)-5α-Spirostan-1β,3β-diol-3-O-{α-L-rhamnopyranosyl-(1→2)-{α-L-rhamnopyranosyl-(1→4)}-[β-D-glucopyranoside} (Table 2)(Figure 1).

Table 2. H-NMR and ^{13}C-NMR data of sugar part of compound (1)
(400 MHz,100 MHz; CD_3OD)

Position	δc (mult)	δH (int,mult, J in Hz), MeOD
I	Glc	
1	104.28(CH)	4.44
2	80.22(CH)	3.84
3	79.58(CH)	3.67
4	79.77(CH)	4.05
5	77.50(CH)	3.44
6	63.75(CH_2)	3.46, 3.65
II	Rh1	
1	104.73(CH)	4.55
2	71.97(CH)	3.93
3	72.47(CH)	3.71
4	75.61(CH)	3.40
5	70.42(CH)	3.93
6	16.41(CH3)	1.15
III	Rh_2	
1	104.96(CH)	4.87
2	72.32(CH)	3.94
3	72.94(CH)	3.74
4	75.88(CH)	3.41
5	71.57(CH)	4.01
6	17.93(CH_3)	1.23

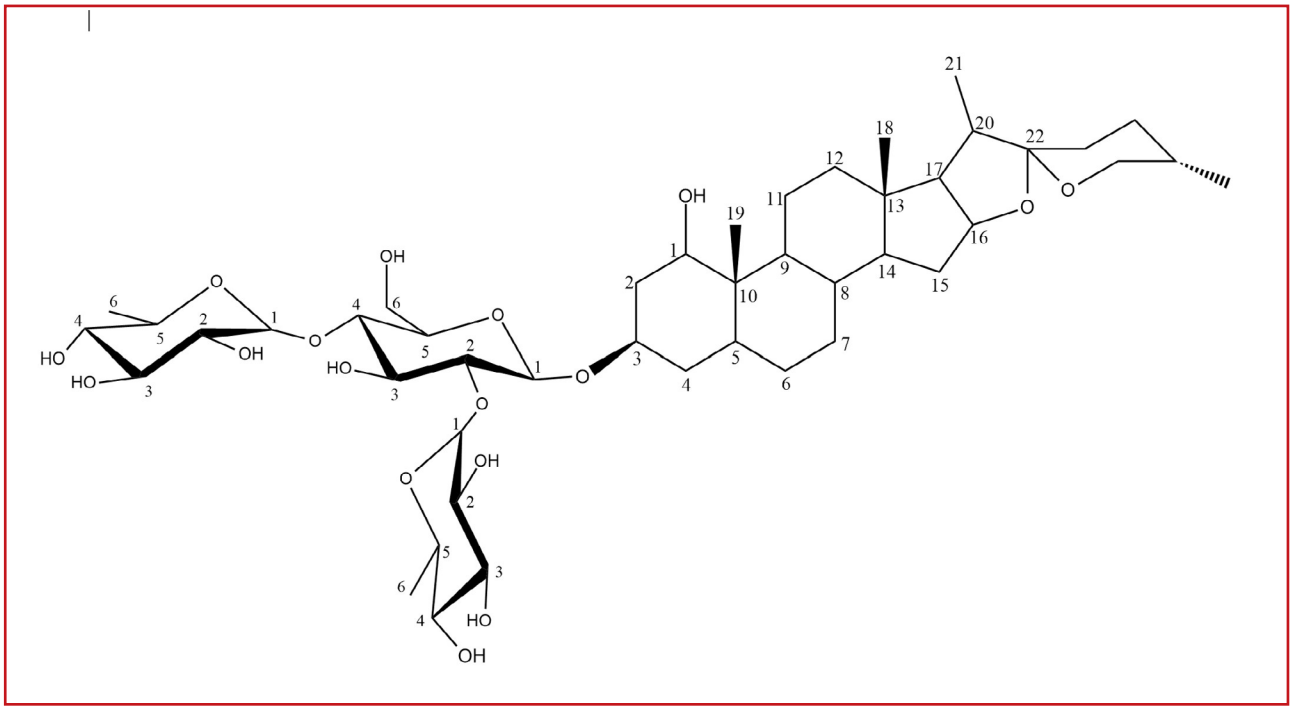


Figure 1. Chemical structure of compound (1) isolated from the flowers of *A. austroiranicum*

Antileishmanial activity of compound (1)

Antileishmanial activity of compound (1) was assessed against the promastigotes of *L. major* using the microplate method (12). As it is shown in Chart 1, compound (1) exhibitedsignificant leishmanicidal activity in 10 ($p< 0.05$), 50 ($p< 0.05$) and 100 ($p< 0.0001$) µg/mL concentrations, during 48 hours of incubation. Although it was not concentration-dependent, in all doses a time-dependent leishmanicidal activity was seen, so the efficacy was increased during 48 hours of incubation.

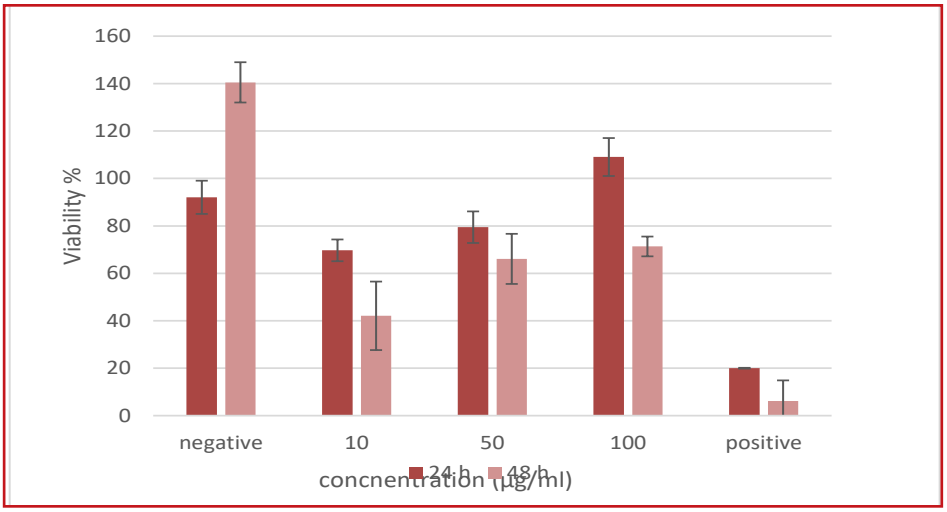


Chart 1. Antileishmanial activities of different concentrations of compound (1) against the promastigotes of *L. major*, 24 and 48 hours after incubation. Results are expressed as mean± SD of the number of viable promastigotes, evaluated by MTT assay. Amphotericin B at concentrations of 100 µg/mL and RPMI medium were used as positive and negative control, respectively

Discussion

As a member of Amaryllidaceae family, *A. austroiranicum* , an important edible *Allium* species in western regions of Iran, has been shown to possess a variety of pharmacological effects including antioxidant activity, antihemolytic activity, hepatoprotective effects and antifungal activity (18, 4).

Phytochemical study of *A. austroiranicum*, aiming the saponin constituents of the plant, resulted in isolation and identification of steroidal saponin from flowers of the plant, using the comprehensive spectroscopic methods, its chemical structure was finally determined as (25S)-5α-Spirostan-1β,3β-diol-3-O-{α-L-rhamnopyranosyl-(1→2)-{α-L-rhamnopyranosyl-(1→4)}-[β-D-glucopyranoside}, commonly named as austrocoside A.

The isolated compound is structurally related to the steroidal saponins isolated from *Allium tuberosum, Allium ampeloprasum* and *Nicotiana tabacum, in* previous studies (20-22).

The spirostan steroid saponins, tuberosides D and M, were previously isolated from the seeds of *Allium* tuberosum, which is widely distributed in Asia and is used as a foodstuff and tonic plant (20).Tuberoside D has a three-unit sugar chain composed of glucose and two rhamnoses, as well as a hydroxyl group at C-2 position, while tuberoside M has a two-unit sugar chain of one glucose and one rhamnose, which is different from the isolated austocoside A in the position of hydroxyl group and nature of sugar chain, respectively. Tuberoside M was shown to possess a significant inhibitory effect on the growth of the human promyelocytic leukemia cell line (HL-60) *in vitro* (23).

While the *Allium ampeloprasum* saponin 1, the spirostanol saponin isolated from *Allium ampeloprasum* (21), has a similar three-unit sugar chain of one glucose and two rhamnoses, it differs in the position of hydroxyl group which is substituted at C-6 position with austrocoside A.

Finally, the Nicotianoside C is a steroidal saponin of the spirostan series which was isolated from the seeds of tobacco *(Nicotiana tabacum L)* (22) and is structurally similar to austrocoside A like three-unit sugar chain (one glucose and two rhamnoses), while it has a different aglycon unit without any OH substitution in the structure.

Considering previous reports on the antimicrobial and especially antileishmanial activity of some natural steroidal saponins, the leishmanicidal effects of the isolated compound were also evaluated. Exhibiting a significant leishmanicidal activity on promastigotes of *L. major*, the results are in line with a few recent reports about the antileishmanial activity of steroidal saponins such as racemoside A, isolated from *Asparagus racemosus* (24), dioscin-related steroidal saponin from *Allium paradoxum* L (12) and spirostanoid fraction from *Allium leucanthum* (25), which could be used as a chemical basis for justification of antimicrobial effects of different *Allium* species and scientific support of future studies of leishmanicidal steroidal saponins.

Conclusion

Phytochemical investigation of *A. austroiranicum* led to the isolation of a new steroidal saponin (Austrocoside A) with significant leishmanicidal activity from the flowers of the plant for the first time, which establishes a valuable basis for further studies about steroidal saponins with anti-parasitic activity. The results are also of great importance for the explanation of biological and pharmacological effects of the plant.

Production of steroidal saponins in very small amounts in plants is one of the limitations of phytochemical study of these compounds, which limits the possibility of obtaining appropriate amounts of the purified compounds for further studies.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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