

Isolation of dioscin-related steroidal saponin from the bulbs of *Allium paradoxum* L. with leishmanicidal activity

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Abstract

Alliums are rich sources of steroidal saponins, flavonoids, and sulphoric compounds of which steroidal saponins have recently received more attention due to their important pharmacological activities. *Allium paradoxum* L. is a common edible vegetable in north regions of Iran, especially in Mazandaran province, where it is named “Alezi” and considerably used as a raw vegetable, to make dishes, and as a medicinal plant. Phytochemical investigation of chloroform-methanol extract of the plant resulted in the isolation and identification of a dioscin related steroidal saponin, using comprehensive spectroscopic methods including 1D and 2D NMR, its chemical structure was determined as (25R)-spirost-5-en-3 β -ol,3-O- α -rhamnopyranosyl-(1 \rightarrow 4)- α -rhamnopyranosyl-(1 \rightarrow 4)-[α -rhamnopyranosyl-(1 \rightarrow 2)]-glucopyranoside. Investigation of *in vitro* antileishmanial activity of the isolated compound, in 10 and 50 μ g/mL concentrations, exhibited significant leishmanicidal effects ($P < 0.001$) against the promastigotes of *Leishmania major*. The results established a valuable basis for further studies about *A. paradoxum* and anti-parasitic activity of steroidal saponins.

Keywords: *Allium paradoxum*; Leishmania; Saponins; Structure elucidation.

INTRODUCTION

The genus *Allium* are very important esculent herbaceous plants compose of about 750 species in 15 subgenera, grow especially in northern hemisphere (1,2). Besides the use in cookery from the ancient times, *Alliums* have been also used as herbal remedies for treatment of many diseases including hypercholesterolemia, hypertension, and diabetes (1,3). In recent decades, many attentions have been attracted to the medicinal effects of these plants and some activities like anti-inflammatory, antispasmodic, antifungal, and antitumor effects have been proved (4).

Phytochemically, *Alliums* are rich sources of steroidal saponins, flavonoids, and organosulfuric compounds (3) of which steroidal saponins have recently attracted more interest due to their important pharmacological activities. Steroidal saponins are naturally occurring glycosides that have some characteristic properties like frothing in aqueous solutions, hemolytic activity, toxicity to fishes, and pharmacological effects

including antifungal, antitumor, cytotoxic, antispasmodic, and cholesterol-lowering activities (1,5).

Allium paradoxum (*A. paradoxum*) which is locally named “Alezi” is a common edible vegetable in north regions of Iran, especially in Mazandaran province. As well as using as a raw vegetable to make dishes, it has some medicinal uses among the local people, especially to regulate the blood cholesterol level, strengthen physical force, and improve digestive and bloodstream system (6). Hepatoprotective (7), renoprotective (8), antihemolytic, and antioxidant activities (9), are other pharmacological effects of the plant which have been proved through the recent studies.

In continuance of our project on phytochemical investigation of *Allium* species, isolation and identification of steroidal saponins from the bulbs of *A. paradoxum* was conducted in the current study.

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According to the antimicrobial and antiparasitic activity of steroidal saponins, the isolated compound was also evaluated *in vitro* for antileishmanial activity.

MATERIALS AND 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 SiO₂ plates (Merck, Germany) with BuOH:H₂O:CH₃COOH (60:25:15 v/v/v) (BAW) as a mobile phase and cerium sulfate in 2N H₂SO₄ 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 and C at 100 MHz) spectrometer, using solvent signal for calibration (CD₃OD: 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 multiple bond correlation (HMBC), optimized for ²⁻³J_{CH} of 8 Hz, was used for determination of two and three bond heteronuclear ¹H-¹³C connectivities, while 2D heteronuclear single-quantum coherence (HSQC), interpulse delay set for ¹J_{CH} of 130 Hz, and correlation spectroscopy (COSY) were used for determination of one-bond heteronuclear ¹H-¹³C connectivities and homonuclear ¹H-¹H connectivities, respectively. Electrospray ionization mass spectroscopy (ESIMS) spectra were prepared by Shimadzu liquid chromatography-mass spectrometry (LCMS) 2010 EV (Japan), using methanol as the solvent.

Plant material

The whole plant of *A. paradoxum* was collected from Babol mountainous areas (Mazandaran, Iran), during April 2014 and identified by the botanist, Mohammad Reza Joharchi. A voucher specimen (No. 2163) 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

Bulbs of *A. paradoxum* were separated, air-dried in the shade, and powdered by means of a mill. The powder (1600 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 maceration method, performing each step four times with 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 (16 g) which was fractionated by MPLC on a RP-18 column (36 × 460 mm) using a linear gradient solvent system of H₂O to CH₃OH. Fractions were analyzed by TLC (SiO₂, BAW 60:15:25 v/v/v) and similar fractions were mixed together. Based on TLC and preliminary NMR analysis, 10th fraction was considered richer in steroidal saponins, which was concentrated by 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 H₂O:CH₃OH (20:80) mobile phase in isocratic mode, resulted the compound (1) (153 mg, t_R = 8 min).

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 4% brain-heart infusion broth (0.2 mL), 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), L- glutamine (2 Mm), 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 Kazemi Oskuee, *et al.* (10). Briefly, *L. major* promastigotes 4×10^5 in 400 µL complemented RPMI were cultured in wells of 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 and 50 µg/mL. The plates were incubated at 25 °C for 2 days and the amount of viable parasites were counted on the time periods of 12, 24, and 48 h. Amphotericin B at concentrations of 100 µg/mL and RPMI medium were used as positive and negative control, respectively.

Statistical analysis

Antileishmanial activities were reported as mean \pm standard deviation (SD) and statically analyzed by one way ANOVA and Tukey-Kramer test (SPSS V. 16). Significant level was considered at $P < 0.05$.

RESULTS

A saponins-riched fraction of the plant extract was selected for further purification, resulted to the isolation and identification of steroidal saponins (**1**) which was structurally related to the famous steroidal saponins, dioscin. 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.

Characterization of compound (1)

The steroidal saponin nature of compound (**1**) was confirmed by ^1H and ^{13}C -NMR spectra of the compound, including those related to steroidal part, a bundle of overlapped signals

at δ_{H} 3 to 5 ppm and the existence of diagnostic and characteristic signals of saponins especially two tertiary methyls (3H singlets: δ_{H} 0.79 and 0.94; C-NMR: δ_{C} 14.89 and 19.85), five secondary methyls (3H doublets; δ_{H} 0.69 ($J = 6.4$ Hz), 0.85 ($J = 7$ Hz), 1.14 ($J = 4.4$ Hz), 1.16 ($J = 4.4$ Hz), 1.20 ($J = 4.4$ Hz); C-NMR: δ_{C} 18, 16.79, 17.87, 17.51, and 18.57), one olefinic proton signal (δ_{H} 5.28) (1H, dd, 5.5, 2.5), four anomeric protons (δ_{H} 4.40, 4.63, 5.30, 5.30) and related anomeric carbon signals (δ_{C} 100.48, 102.63, 102.39, and 103.21) (Table 1). In the ESIMS spectra, compound (**1**) showed a pseudomolecular ion peak at m/z 1013.5 [M-H] in the negative-ion mode that together with the ^{13}C -NMR data, suggested its molecular formula as $\text{C}_{51}\text{H}_{82}\text{O}_{20}$. Using the mass spectrometry (MS) and NMR spectral data and comparing them with those reported in the literature (11,12), the nature of the aglycon part of the compound was determined as diosgenin, which finally was confirmed by HMBC and COSY correlations.

To deduce the glycon part of the compound (**1**), starting from the first anomeric proton ($\text{H}1^{\text{I}}$; δ_{H} 4.4) and using the HSQC and COSY spectral data, specially the characteristic large coupling constant of $\text{H}1^{\text{I}}$, the first sugar was determined as β -glucopyranoside. Doing the same type of analysis for other three sugars resulted in the identification of three α -rhamnopyranoyl sugars and completion of sugar chain structure elucidation (Table 2). The sequence of sugars connectivity to each other and also to the aglycon was determined by HMBC correlations. According to the HMBC cross peaks of $\text{H}1^{\text{I}}\text{-C}3$ (δ_{H} 4.40 - δ_{C} 77.97) and $\text{H}3\text{-C}1^{\text{I}}$ (δ_{H} 3.46 - δ_{C} 100.48), the glucose residue was concluded to be attached to the C3 of the aglycon, while the position of remaining rhamnose residues in the sugar side chain was determined through the cross peaks of $\text{H}1^{\text{II}}\text{-C}2^{\text{I}}$ (δ_{H} 5.30 - δ_{C} 80.88), $\text{H}1^{\text{III}}\text{-C}4^{\text{I}}$ (δ_{H} 4.63 - δ_{C} 79.53), and $\text{H}1^{\text{IV}}\text{-C}4^{\text{III}}$ (δ_{H} 5.30 - δ_{C} 76.70). This was further confirmed by the glycosilation shifts of $\text{C}2^{\text{I}}$, $\text{C}4^{\text{I}}$, and $\text{C}4^{\text{III}}$ and also by the fragmentation peaks in the ESIMS spectrum due to the loss of sugar units from the pseudomolecular ion, e.g. 866.5 [M-H-147] and 720.5 [M-H-147-146].

Table 1. ¹H-NMR and ¹³C-NMR data of aglycon part of compound (1) (400 MHz, 100 MHz; CD₃OD)

Position	δ_H (int., mult., J in Hz)	δ_C (mult)
1a,b	0.96 ^a , 1.79 ^a	38.57 (CH ₂)
2a,b	1.32 ^a , 1.51 ^a	30.76 (CH ₂)
3	3.46 ^a	77.97 (CH)
4a,b	2.21 ^a , 2.25 ^a	39.50 (CH ₂)
5	-	141.90 (C)
6	5.28 (1H, dd, 5.5, 2.5)	122.67 (CH)
7a,b	1.79 ^a , 1.82 ^a	32.80 (CH ₂)
8	1.54 ^a	32.42 (CH)
9	0.86 ^a	51.72 (CH)
10	-	38.05 (C)
11a,b	1.45 (1H, dd, 10.5, 2.5), 1.39 (1H, m)	21.99 (CH ₂)
12a,b	1.64 ^a , 1.09 ^a	40.94 (CH ₂)
13	-	41.43 (C)
14	1.03 ^a (1H, m)	57.81 (CH)
15a,b	1.18 ^a , 1.88 ^a	32.74 (CH ₂)
16	4.28 (1H, q, 7.2)	82.24 (CH)
17	1.64 ^a	61.92 (CH)
18	0.79 (3H, s)	14.89 (CH ₃)
19	0.94 (3H, s)	19.85 (CH ₃)
20	1.80 ^a	42.92 (CH)
21	0.85 (3H, d, 7)	16.79 (CH ₃)
22	-	110.64 (C)
23a,b	1.59 ^a , 1.47 ^a	33.19 (CH ₂)
24a,b	1.50 ^a , 1.23 ^a	29.88 (CH ₂)
25	1.18 ^a	31.44 (CH)
26a,b	3.33 ^a , 3.21 ^a	67.88 (CH ₂)
27	0.69 (3H, d, 6.4)	18.00 (CH ₃)

(a) Overlapped with other signals.

Table 2. ¹H-NMR and ¹³C-NMR data of sugar part of compound (1) (400 MHz, 100 MHz; CD₃OD)

Position	δ_C (mult)	δ_H (int, mult, J in Hz)
I	Glc	
1	100.48 (CH)	4.40 (1H, d, 7.8)
2	80.88 (CH)	3.85 ^a
3	79.45 (CH)	3.67 ^a
4	79.53 (CH)	4.10 ^a
5	77.29 (CH)	3.45 ^a
6	63.72 (CH ₂)	3.47 ^a , 3.65 ^a
II	Rh₁	
1	102.39 (CH)	5.30 (1H, bs)
2	72.16 (CH)	3.89 ^a
3	72.20 (CH)	3.72 ^a
4	73.85 (CH)	3.40 ^a
5	69.81 (CH)	3.89 ^a
6	17.51 (CH ₃)	1.16 (3H, d, 4.4)
III	Rh₂	
1	102.63 (CH)	4.63 (1H, bs)
2	72.93 (CH)	3.91 ^a
3	72.96 (CH)	3.72 ^a
4	76.70 (CH)	3.40 ^a
5	69.09 (CH)	4.1 ^a
6	18.57 (CH ₃)	1.20 (3H, d, 4.4)
IV	Rh₃	
1	103.21 (CH)	5.30 (1H, bs)
2	72.38 (CH)	3.89 ^a
3	72.42 (CH)	3.72 ^a
4	73.94 (CH)	3.40 ^a
5	70.47 (CH)	3.89 ^a
6	17.87 (CH ₃)	1.14 (3H, d, 4.4)

(a) Overlapped with other signals.

On the basis of these data, the chemical structure of compound (1) was determined as (25R)- spirost-5- en- 3 β -ol,3-O- α -rhamnopyranosyl-(1 \rightarrow 4)- α -rhamnopyranosyl -(1 \rightarrow 4)-[α -rhamnopyranosyl-(1 \rightarrow 2)]- glucopyranoside, a steroidal saponin analogous to dioscin (11,12) with an additional rhamnose in the sugar chain (Fig. 1).

Antileishmanial activity of compound (1)

Antileishmanial activity of compound (1) was assessed against the *L. major* promastigotes using the microplate method. As

it is shown in Fig. 2, compound (1) exhibited significant leishmanicidal activity in both 10 and 50 μ g/mL concentrations. In 10 μ g/mL, compound (1) exhibited a significant leishmanicidal activity in 12 h ($P < 0.001$) and 24 h ($P < 0.0001$) which increased over the time and reached to its maximum activity in 48 h after incubation, when it eradicated the leishmania promastigotes completely. Similarly, at 50 μ g/mL, considerable leishmanicidal activity was observed in 12 h ($P < 0.0001$) reaching to its maximum effect and full eradication of promastigotes in 24 h.

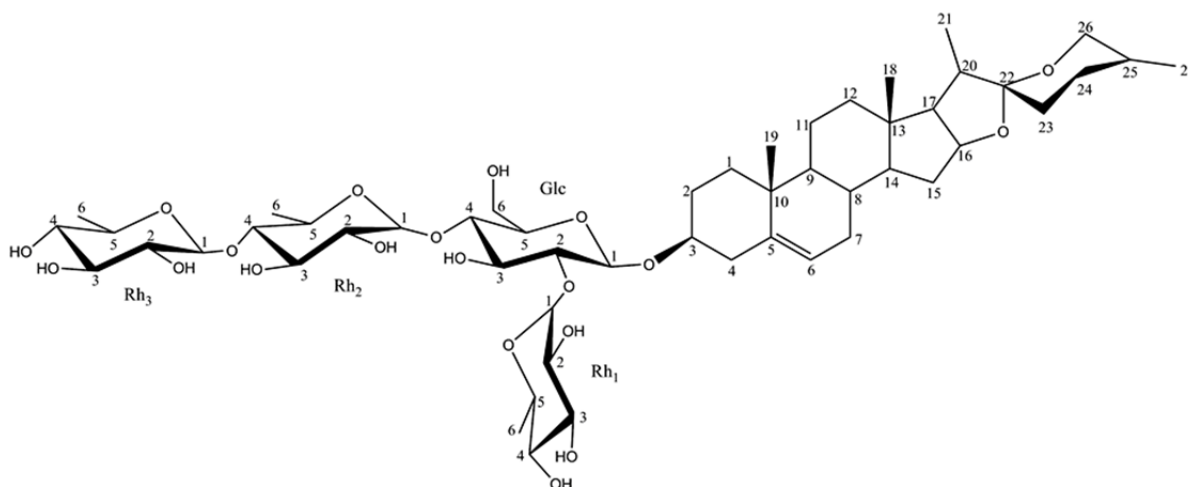


Fig. 1. Chemical structure of compound (1) isolated from the bulbs of *A. paradoxum*.

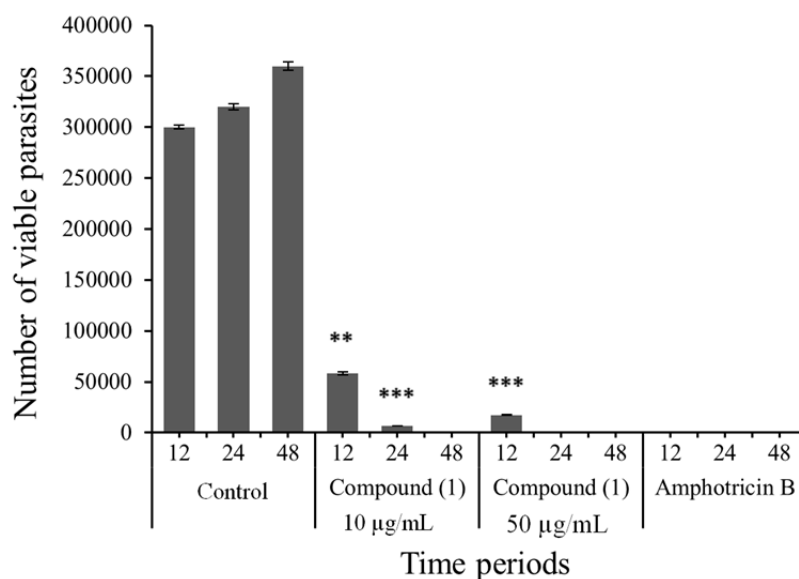


Fig. 2. Antileishmanial activities of different concentrations of compound (1) and amphotericin B against *L. major* promastigotes, 12, 24, and 48 h after incubation. Results are expressed as mean \pm SD of the number of viable promastigotes in 12 wells. ** $P < 0.001$ and *** $P < 0.0001$ against the control.

DISCUSSION

As a member of Alliaceae family, *A. paradoxum*, an important edible *Allium* species in northern regions of I.R. Iran, has been shown to possess a variety of pharmacological effects including antioxidant activity, antihemolytic activity, hepatoprotective effects against liver toxicity induced by CCl₄, and protective effects against gentamicin-induced nephrotoxicity (7,8,9). Phytochemical study of *A. paradoxum*, specially the saponin constituents of the plant, resulted in isolation and identification of a dioscin related steroidal saponin from bulbs of the plant, which is in agreement with previous studies including isolation of this compound from *Allium ursinum* (11). Considering previous reports on the antimicrobial and specially antileishmanial activity of some natural steroidal saponins, the leishmanicidal effects of the isolated compound was evaluated, which interestingly exhibited its significant leishmanicidal activity on promastigotes of *L. major*. The results are in line with few recent reports about the antileishmanial activity of some steroidal saponins such as racemoside A, isolated from *Asparagus racemosus* (13), 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.

Dioscin is probably the most famous naturally occurring steroidal saponin which has been isolated from a variety of different plant species; of which the plant species of Dioscoreaceae family are more important due to their medicinal and pharmaceutical applications (14,15). Besides the use as the starting material for industrial synthesis of steroidal drugs in pharmaceutical industries, especially as diosgenin containing compound (the aglycone part of dioscin) (16,17), dioscin have been also reported to possess numerous pharmacological effects including cytotoxic effects through the induction of apoptosis (18), antitumor, antifungal (16), inhibition of bone resorption and osteoclast differentiation (19), anti-inflammatory, lipid-lowering, and hepatoprotective activities (15).

CONCLUSION

Phytochemical investigation of *A. paradoxum* led to the isolation of a steroidal saponin with significant leishmanicidal activity from 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 explanation of biological and pharmacological effects of the plant.

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