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Anti-schistosomiasis triterpene glycoside from the Egyptian medicinal plant *Asparagus stipularis*[#]

Hesham R. El-Seedi,[*,1,2,7] Rehan El-Shabasy,[2] Hanem Sakr,[2] Mervat Zayed,[2] Asmaa M. A. El-Said,[2] Khalid M. H. Helmy,[2] Ahmed H. M. Gaara,[3] Zaki Turki,[4] Muhammad Azeem,[1] Ahmed M. Ahmed,[5] Loutfy Boulos,[6] Anna-Karin Borg-Karlson,[1] Ulf Göransson[7]

[1]Department of Chemistry, Royal Institute of Technology KTH, Sweden,
[2]Department of Chemistry, Faculty of Science, El-Menoufia University, Egypt,
[3]Chemistry of Natural and Microbial Products, National Research Centre, Egypt,
[4]Department of Botany, Faculty of Science, El-Menoufia University, Egypt,
[5]Department of Botany, Desert Research Centre, Egypt,
[6]Department of Botany, Faculty of Science, Alexandria University, Egypt,
[7]Division of Pharmacognosy, Department of Medicinal Chemistry, Uppsala University, Sweden.

Abstract: Bioassay-guided isolation using an *in vitro* assay testing for anti-schistosomiasis yielded a novel triterpene saponin, asparagalin A, from the *n*-butanol extract of the roots of *Asparagus stipularis* Forssk., Asparagaceae. The structure was elucidated by spectroscopic analysis and chemical transformations. Administration of asparagalin A resulted in a retardation of worm growth and locomotion at the first day and showed a significant activity of egg-laying suppression at 200 µg/mL concentration.

Article

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Introduction

Schistosomiasis (bilharziasis) is a severe, debilitating disease caused by infection with trematodes of the genus *Schistosoma.* Approximately 200 million people are affected worldwide (Chitsulo et al., 2000). *S. haematobium* and *S. mansoni* are widely distributed in Africa and the Middle East. The raising costs of chemotherapy and synthetic molluscicides have led to an increasing interest in plants, which are lethal to the intermediate host of schistosomiasis (Hostettmann, 1984). Plants of the *Asparagus* genus are known for its medicinal utility. Alkaloids, isoflavonoids and steroidal glycosides have been reported from *A. gobicus* and *A. racemosus* (Yang et al., 2004; Mandal et al., 2006).

Asparagus stipularis Forssk., Asparagaceae, commonly known in Egypt as 'agool gabal', is short rootstocks-producing aerial annual or perennial plant with branching stems (Täckholm, 1974). Bedouins in the Mediterranean region use the infusion of the tuberous roots of the plant to remove renal stones, cure syphilis and against headache. Decoction of whole plant diaphoretic young tender used to relieve stomach ache and promote appetite an effect similar to berries, shoots and roots, as diuretic, for jaundice, liver ailments, against bilharzias and rheumatism. The plant fried with eggs and camel's fat to work as spermatogenesis, facilitate secretions and releases obstructions. Seeds are decocted for haemorrhoids (Boulos, 1983).

Earlier phytochemical investigators disclosed the isolation of steroidal glycosides (Halim et al., 1989) but no pharmacological investigation of the studied species is as reported up to now. The plants were collected in Egypt and investigated as a part of our ongoing chemical and pharmacological studies of plants used in folk medicine (Bruhn et al., 2002; El-Seedi, 2007; Bruhn et al., 2008; El-Seedi et al., 2010). In this paper, we investigated the species *A. stipularis*; with the objective to isolate, purify and elucidate the active chemical constituent(s) based on the activity shown by the extract.

Material and Methods

Plant material

Roots of *Asparagus stipularis* Forssk., Asparagaceae, were collected in July 2001 from Sinai and identified by Prof. A. M. Ahmed, Botany Dept., Desert Research Centre, Cairo, Egypt and Dr. Z. Turki, Botany Department, Faculty of Science, El-Menoufia University,

[#]Dedicated with regards and respect to Prof. Shosuke Yamamura, formal Professor of Organic Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan. on the occasion of his 75th birthday

Egypt. A voucher specimen, HRE 1, is deposited in the Herbarium Division, Department of Botany, Faculty of Science, El-Menoufia University, Shebin El-Kom, Egypt.

Extraction and isolation

The roots (2.64 kg) were extracted exhaustively (at room temp.) three times with 70% EtOH (8 l) using mixer. The extracts were evaporated in vacuum to give 89.5 g of a semisolid material. The extract was partitioned between H_2O and *n*-BuOH. The *n*-BuOH layer was washed with H_2O and distilled under reduced pressure to give 60.6 g. The fraction (59 g) was dissolved in a minimum vol. of MeOH and chromatographed on silica gel (120 g) using SEPARO MPLC equipment as mentioned below. Fractions (200 mL) were monitored by TLC (silica gel; $CHCl_3$:EtOAc:MeOH:H_2O 75:10:14:1). The spots on the TLC plates were visualized by spraying with L.B. reagent and the same repeated with vanillin-sulphuric acid followed by heating. The fractions were collected after TLC inspection and ^{1}H-NMR analysis to give eighteen fractions, which were subjected to *in vitro* anti-schistosomal biological activity. Fractions 14 and 15 showed high activity and were further purified by repeated SEPARO MPLC column using $CHCl_3$-MeOH (90:10, 80:20, 60:40, 20:80) as a mobile phase followed by purification by Sephadex LH-20 on CC using the same eluent and finally by prep. TLC using $CHCl_3$:EtOAc:MeOH:H_2O (75:10:14:1) which yielded 18 mg of a pure compound, named asparagalin A (**1**).

Experimental procedures

NMR spectra were recorded on Varian VXR-400 at 25° using CD_3OD as solvent and all chemical shifts are expressed with reference to TMS. ^{1}H-NMR, HMBC, HMQC, COSY, TOCSY and NOESY were obtained at 400 MHz. ^{13}C- and DEPT-NMR spectra were recorded at 101 MHz. TLC was performed on pre-coated Merck aluminium sheets (Silica 60 F254), detection provided by UV at 254 nm and spraying with vanillin-sulphuric acid followed by heating (120°).

MPLC was performed using SEPARO AB MPLC equipment (Baeckström SEPARO AB, Lidingö, Sweden) which also referred as accelerating gradient chromatography (AGC) with variable-length glass columns with inner diameters of 4 cm, packed with silica gel 60, 40-63 µm (Merck), were used. An FMI Lab pump, model QD (Fluid Metering Inc., Oyster bay, NY) was used at a flow rate of 18-28 mL/min. Fractions of 18 mL was collected with a Gilson Model 201 fraction collector. The columns were eluted with continuous gradients running from hexane, over $CHCl_3$, over EtOAc to MeOH and H_2O afforded by a SEPARO constant-volume mixing chamber combined with an open reservoir. Initially, the mixing chamber contained 50 mL non-polar solvent and the reservoir contained the first of 15-20 premixed binary (less polar/more polar solvent) gradient mixtures, of 200-400 mL each, which were successively fed to the reservoir during the separation (Jiron, 1996).

Saponin test

About 2 g of the powdered sample was boiled in 20 mL of distilled water in a water bath and filtered. A 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

*Asparagalin A (**1**)*

White amorphous powder. M.p. 199-201°. $[\alpha]_D^{22}$ = +5.7° (*c*=0.05 mg/mL, MeOH). *UV*λ_{max}^{MeOH} nm (log ε): 210 (4.40). *IR*ν_{max}^{KBr} cm^{-1}: 3431 (OH), 1630 (-C=C-H). ^{1}H- and ^{13}C-NMR (CD_3OD) spectra: see Table 1 and 2. HREIMS *m/z* 1036.4959 $[M]^+$ (calcd. for $C_{49}H_{80}O_{21}S$, 1036.4913).

Acid hydrolysis of asparagalin A

The compound **1** (5 mg) was refluxed on a water bath for eight hrs with 4 N HCl in MeOH (15 mL). The acid hydrolysate was concentrated, extracted with EtOAc. The acidic mother liquor was neutralized with Na_2CO_3, filtered, and evaporated to dryness for examination of the sugar moiety, which proved to be β-D-glucose, β-D-galactose and α-L-rhamnose by detection on TLC [EtOAc-isopropanol- H_2O (65:23:12)], and sprayed with freshly prepared anisaldehyde-H_2SO_4 reagent followed by heating (Stahl, 1962). The sugars were also confirmed by optical rotation as β-D-glucose: $[\alpha]_D^{22}$ =+19.7° (*c*=0.45 mg/ml, H_2O); α-L-rhamnose: $[\alpha]_D^{22}$ =+3.6° (*c*=0.45 mg/ml, H_2O); β-D-galactose: $[\alpha]_D^{22}$ =+8.1° (*c*=0.45 mg/ml, H_2O).

In vitro *anti-schistosomal activity*

The test was assayed using the method reported previously by Ohigashi et al. (1994). Briefly, adult pair of schistosomes of Egyptian strain *S. mansoni* was cultured in RPMI-1640 medium. After incubation for 24 h, inhibition of both the movement and egg-laying capability of the schistosomes (Mortality rate) in triplicate experiments was evaluated by four ranks; (+++) corresponding to 75-100% inhibition during the 1st day while 51-74% activity during the 2nd day classified as moderately active (++),<50% are weakly active by (+) and no effect (-) described as inactive according to the percentage of mortality. Commercially available praziquantel was used as a positive control.

Results and Discussion

Using *in vitro* assay guiding for inhibition of schistosomiasis, an *n*-butanol soluble fraction of a MeOH extract from the roots of *A. stipularis* was fractionated mainly by combining MPLC, CC and prep. TLC on silica gel, and Sephadex LH-20 chromatographic techniques. The fractionation procedure followed by successive elution $CHCl_3$, EtOAc, EtOAc-MeOH, MeOH and MeOH-H_2O resulted in the isolation and structure determination of a novel dammarane glycoside named asparagalin A (**1**). The aglycone of asparagalin A consists of a dammarane skeleton glycosidically bound at C-3 to three sugars. To the best of our knowledge, this is the first report of dammarane-type triterpenoid saponins from the family Liliaceae.

The compound **1**, which was isolated as an amorphous powder, gave a positive forth test for saponins, a positive Libermann-Burchard (L.B.) test for triterpenes and a positive Molisch test for sugars.

The high-resolution FAB-MS spectrum showed a molecular ion peak at *m/z* 1036.4959 suggesting the molecular formula $C_{49}H_{80}O_{21}S$. The ^{13}C- and DEPT-NMR spectra showed 49 signals, of which eighteen were assigned to the saccharide portion and 31 to a terpenoid moiety. The ^{1}H- and ^{13}C-NMR spectra (Table 1, 2) showed anomeric centre at δ_C 105.8 and δ_H 4.31 (*d*, *J*=8) and δ_C 105.8, δ_H 5.44 (*br s*) and δ_C 101.8 and δ_H 4.40 (*d, J*=7.5) and δ_C 105.2, respectively, which indicated the presence of a trisaccharide moiety. An unambiguous determination of the sugar sequence and sites of linkage were detected from the correlations deduced from the COSY, TOCSY, NOESY and HMBC spectra. Thus, the NOESY spectrum showed correlation between H-1′ at δ 4.31 and H-3 at δ 3.16, while the HMBC spectrum displayed cross-peaks between H-1′ at δ 4.31 and the aglycone C-3 at δ 89.9 proving that glucose unit was linked to C-3 of the aglycone. Also, clear correlation of H-1″ at δ 5.44 and C-2′ at δ 75.1 and correlation of H-1‴ at δ 4.40 and C-3′ at δ 78.0. Similarly, the same conclusion was also drawn from the NOESY spectrum. TLC and comparison with authentic samples also confirmed the sugar parts after hydrolysis. The aglycone moiety was unambiguously assigned as dammarane-type based on ^{1}H- and ^{13}C-NMR spectra and homo- and hetero-nuclear correlations observed in COSY, HMBC, and HMQC, as well as through comparison with literature data (Kurihara et al., 1988). The ^{1}H-NMR spectrum showed six signals assignable to tertiary methyl groups at δ 0.86-1.64 and one signal assignable to a secondary methyl group at δ 1.04, in addition to two characteristic olefinic protons at δ 4.72, which are in agreement with literature data. The structure of the side chain of the aglycone moiety was unambiguously established by 2D NMR. From the H-H COSY spectrum correlation cross peak between the methyl group at δ 1.04 and one proton at δ 2.38 (CH), furthermore HMBC (Figure 1) showed clear correlation of H-27 at δ 4.72 and C-28, 26, 24 at δ 18.6, 149.9, 39.6, respectively confirming the side chain as CH_3-CH-C(CH_3)=CH_2. All together, the data and a comparison with closely related compounds (Renault et al., 1977; Ikram et al., 1981; Maciuk et al., 2004) agree with the formula shown in **1** for the novel triterpene saponin, for which we propose the trivial name asparagalin A.

Complete inhibition of the worm movement was detected in this study of fractions 14 and 15, when schistosomes were treated with the test compound for 24 h gave (+++) which considered as highly active. Accordingly, they were further purified to give asparagalin A. Asparagalin A was evaluated for its effect on the worm egg-laying capacity. The number of eggs was counted and divided by the number of worm pairs to give the mean number of eggs per worm pair. Microsoft Excel was used to calculate the geometric mean and the standard error (SE). The mean number of eggs at different concentrations of asparagalin A (200, 20 and 2 µg/ml) are shown in (Table 3). It is clear from the results that there was suppression of egg-laying capacity in a dose-dependence manner.

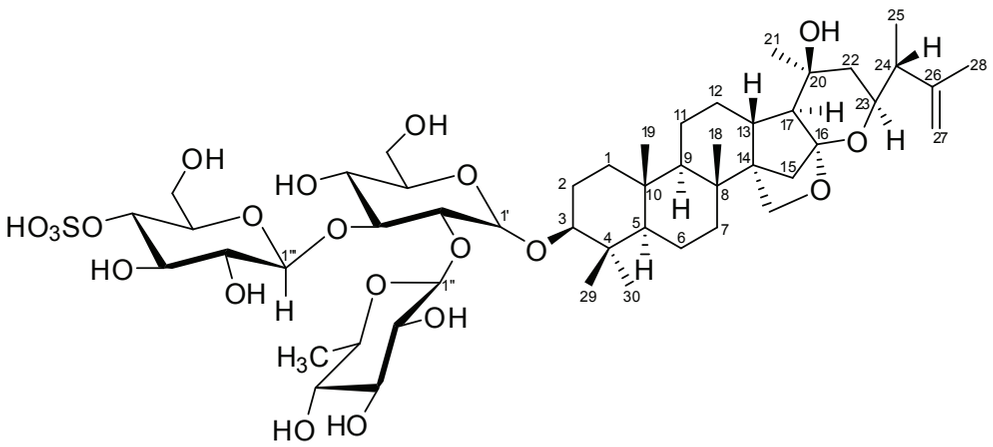

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Table 1. ^{1}H- and ^{13}C-NMR chemical shifts of the aglycone asparagalin A (**1**) in CD_3OD; δ in ppm.

Position	^{1}H	^{13}C
1	0.95, 1.65*	40.1 (*t*)
2	1.65*, 1.95	27.4 (*t*)
3	3.16	89.9 (*d*)
4		40.5* (*s*)
5	0.73	57.6 (*d*)
6	1.54	18.9 (*t*)
7	1.64	36.9 (*t*)
8		38.7 (*s*)
9	0.73	53.8* (*d*)
10		38.2 (*s*)
11	1.55	22.2 (*t*)
12	1.60, 1.80	28.2 (*t*)
13	2.38	38.1 (*d*)
14		57.5 (*s*)
15	1.39, 1.99	38.9 (*d*)
16		119.2 (*s*)
17	1.47	53.8* (*d*)
18	1.08	19.2 (*q*)
19	0.86	16.9 (*q*)
20		76.6 (*s*)
21	1.12	23.2 (*q*)
22	1.50	40.5* (*t*)
23	4.71	66.8 (*d*)
24	2.38	39.6 (*d*)
25	1.04	21.3 (*q*)
26		149.9 (*s*)
27	4.72	111.3 (*t*)
28	1.64	18.6 (*q*)
29	0.86	17.1 (*q*)
30	1.05	28.4 (*q*)
31	4.06	66.7 (*t*)

(*) Overlapping signals

It is noteworthy that dammarane-type triterpenoid saponins are major constituents of a number of reputed herb drugs including ginseng (Garai et al., 1996). Although this type of compounds has been isolated from some medicinal plants of different families (*e.g.* Rhamnaceae and Scrophulariaceae); to the best of our knowledge, this is the first report from the family Liliaceae. On the other hand, our findings agree with the fact that saponins have been emphasized as molluscicidal compounds of plant origin (Webbe & Lambert, 1983).

Table 2. ^{1}H and ^{13}C-NMR assignments of the sugar moieties of asparagalin A (**1**) in CD_3OD δ in ppm, *J* (Hz).

Position	^{1}H	^{13}C
β-D-glucose		
1	4.31 (*d*, *J* = 8)	105.8
2	3.20 (*dd*, *J* = 8.5, 7)	75.1
3	3.34 (*t*, *J* = 9)	78.0
4	3.27 (*t*, *J* = 9)	71.2
5	3.40 (*m*)	77.9
6	3.61 (*dd*, *J* = 11, 6) 3.88 (*d*, *J* = 11)	62.4
α-L-rhamnose		
1	5.44 (br *s*)	101.8
2	3.97 (*dd*, *J* = 3.5, 1.5)	72.1
3	3.73 (*dd*, *J* = 9.5, 3.5)	72.1
4	3.39 (*t*, *J* = 9.5)	73.8
5	4.00 (*m*)	70.4
6	1.22 (*d*, *J* = 6)	17.9
β-D-galactose		
1	4.40 (*d*, *J* = 7.5)	105.2
2	3.84 (*dd*, *J* = 9, 7.5)	72.2
3	3.77 (*dd*, *J* = 9, 2.5)	70.0
4	4.13 (br *d*, *J* = 3)	85.9
5	3.52 (*t*, *J* = 6)	75.7
6	3.72-3.74 (*m*)	62.2

Table 3. Effect of asparagalin A on *S. mansoni* egg-laying capacity.

Concentration	200 µg/mL	20 µg/mL	2 µg/mL
Mean number of eggs	27±18	127±99	822±85

All data are expressed as mean±SE.

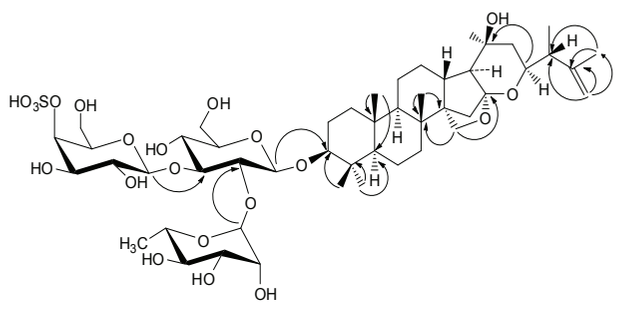

Figure 1. Important ^{1}H-^{13}C-couplings in the HMBC spectrum of asparagalin A (**1**).

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***Correspondence**

Hesham R. El-Seedi
Division of Pharmacognosy, Department of Medicinal Chemistry, Uppsala University, Biomedical Centre, Box 574, SE-751 23, Uppsala, Sweden
hesham.el-seedi@fkog.uu.se
Tel.: +46 18 4714207
Fax: +46 18 509101