Identification of sulfated steroidal glycosides from the starfish *Heliaster helianthus* by electrospray ionization mass spectrometry

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Dedicated to Professor Atta-ur-Rahman on his 65th anniversary

Abstract

The starfish *Heliaster helianthus* contains Thornasteroside A (**1**) and Helianthoside (**2**) and its isomer (**3**) as the major sulfated steroidal glycosides. Saponin containing fractions obtained by purification of extracts of *H. helianthus* induced escape reactions and mortality in the limpets *Siphonaria lessoni* and *Fissurella radiosa*. ESI-MS and ESI-MS/MS were successfully applied to the rapid screening of two cytotoxic fractions obtained by purification of the ethanolic extracts of arms and central disks dissected from *H. helianthus*. Three major compounds were identified, together with minor sulfated steroidal mono- and diglycosides of polyhydroxysteroids.

Keywords: Starfish, *Heliaster helianthus*, sulfated glycosides, electrospray ionization tandem mass spectrometry

Introduction

Sulfated steroidal oligoglycosides (asterosaponins) have been recognized as predominant and characteristic metabolites of starfishes.[1] Asterosaponins, which are responsible for the toxicity of these marine organisms, are accompanied by a variety of steroidal mono- and diglycosides and by free and sulfated polyhydroxysteroids in very complex mixtures of highly oxygenated compounds. Starfish extracts have drawn attention because of their wide spectrum of biological

effects associated with antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory activities.[2] Recently, we have demonstrated the antifungal activity of two new sulfated hexaglycosides and two new sulfated polyhydroxylated steroidal xylosides isolated from the Patagonian starfish *Anasterias minuta*.[3]

In our continuing search for bioactive metabolites from echinoderms and aiming to elucidate the possible biological role of steroidal glycosides in starfish, we have focused our attention on the polar extracts of the limpet predatory starfish *Heliaster helianthus* (Lamarck, 1816), collected off Las Cruces, Chile. Previous work on this starfish led to the isolation of the common pentaglycoside Thornasteroside A (**1**) together with the sulfated polyhydroxylated steroidal xylosides Helianthoside (**2**) and its isomer (**3**) (Fig. 1).[4,5]

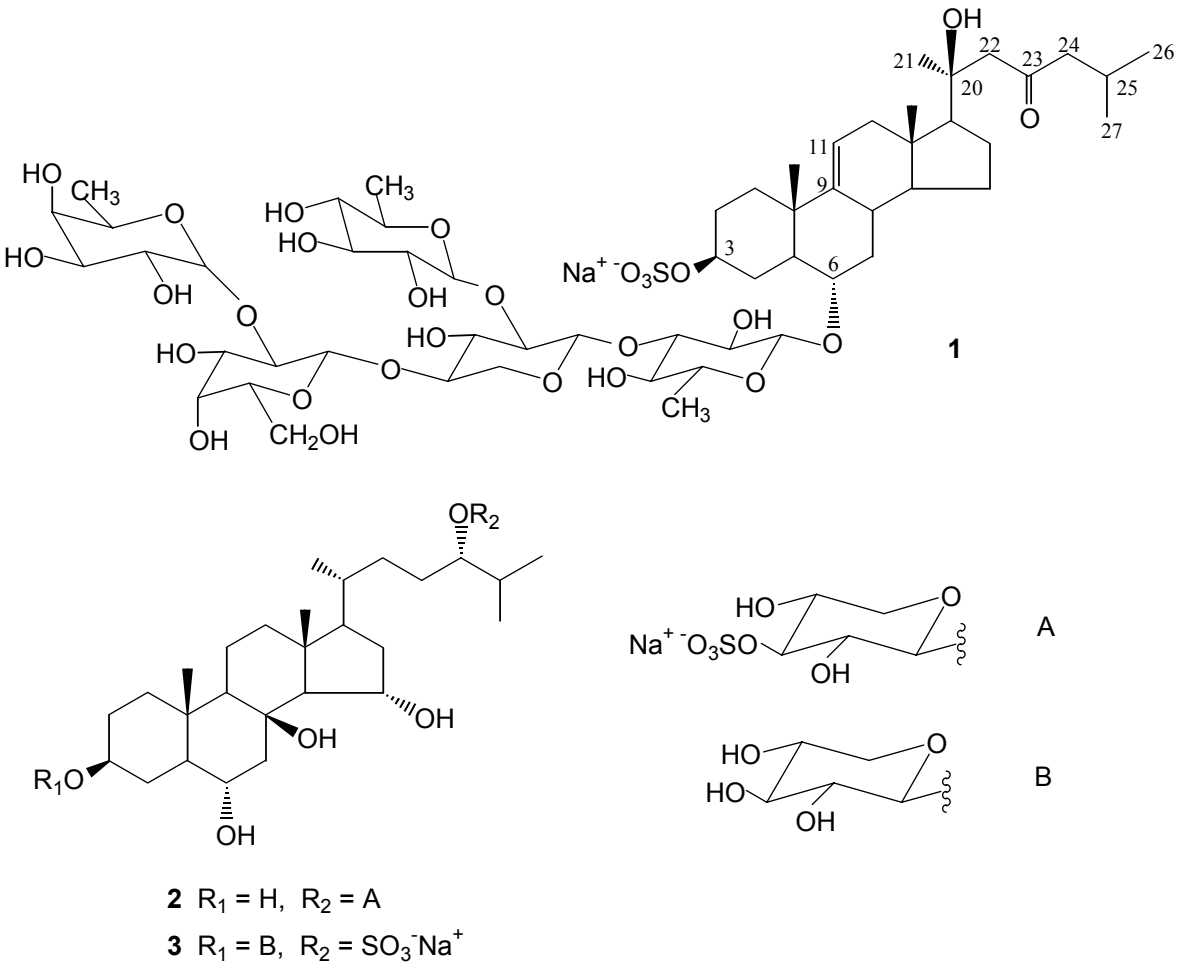

Figure 1. Chemical structures of sulfated steroidal glycosides **1-3**.

Previous field experiments demonstrated that the limpets *Lottia orbignyi* and *Scurria viridula* display active locomotor escape response following contact with homogenates of *H. helianthus*.[6] Recently, we observed that saponin containing fractions obtained by purification of extracts of

H. helianthus induce escape reactions and mortality in the limpets *Siphonaria lessoni* and *Fissurella radiosa*.[7] To correlate the results of these field experiments with the saponin composition of the tested fractions, we used direct infusion electrospray ionization mass spectrometry as a rapid analytical tool to identify the bioactive compounds in the fractions. Mass spectrometry has played an important role in the structural analysis of natural products owing mainly to its high sensitivity, selectivity and low levels of sample consumption. With the development of soft ionization techniques such as electrospray ionization (ESI), rapid and direct analysis of polar, non-volatile and thermally labile classes of compounds has been achieved.[8] Recently, ESI tandem mass spectrometry (ESI-MS/MS) has been shown to provide useful structural information on saponins in plant extracts.[9]

In the present work, we employed ESI mass (ESI-MS) and tandem mass spectrometries (ESI-MS/MS) for analysis of the polar steroidal glycosides responsible of the toxicity of the purified fractions of the extract of *H. helianthus*.

Results and Discussion

The *n*-BuOH extracts of the arms and central disks of *H. helianthus* exhibited cytotoxicity in the brine shrimp (*Artemia salina* L.) larvae mortality bioassay.[10] Purification of both extracts by chromatography over Sephadex LH60 and further analysis by TLC and comparison with standards led to the isolation of fractions rich in polar steroidal oligoglycosides together with fractions containing predominantly steroidal monoglycosides. Two of these fractions (F4 and F8) were selected for ESI-MS studies to obtain molecular mass and structural information on the starfish steroidal glycoside mixtures.

Fraction F4 was obtained by purification of the *n*-BuOH extract of the arms and contained asterosaponins as the major components. The ESI-MS in the negative ion mode of this fraction (Fig. 2) displays an ion of *m/z* 1243 assigned to the corresponding anion $[M - Na]^-$ of Thornasteroside A (**1**), the only asterosaponin reported so far from *H. helianthus*.[4]

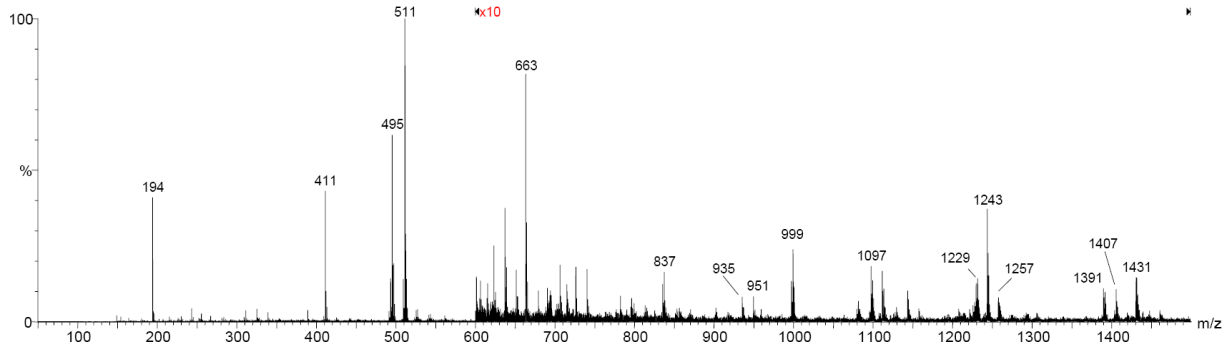

Figure 2. ESI-MS of fraction F4 in the negative ion mode.

The ESI-MS/MS of the isolated ion of *m/z* 1243 (Fig. 3a) displays a fragment ion of *m/z* 1143 corresponding to the loss of a $C_6H_{12}O$ molecule arising from C(20)-C(22) bond cleavage and 1H transfer (retro aldol cleavage), characteristic of asterosaponins containing an aglycone with a 20-hydroxy-23-oxo side chain.[11] The major ion of *m/z* 511 in the ESI-MS of fraction F4 (Fig. 2) corresponds to the fragment ion of the sulfated aglycone of asterosaponin **1**. ESI-MS/MS experiment of the isolated ion of *m/z* 511 (Fig. 3b) shows it to fragment to an ion of *m/z* 411 due to retro aldol cleavage of the aglycone side chain (loss of $C_6H_{12}O$) and to a fragment ion of *m/z* 97 attributed to SO_4H^-. In the ^{13}C-NMR spectrum of fraction F4 signals at δ 13.8 (C-18), 22.7 (C-26), 22.9 (C-27), 27.3 (C-21), 74.0 (C-20), 77.8 (C-3), 80.4 (C-6), 116.7 (C-11), 146.5 (C-9) and 211.9 (C-23) ppm are consistent with the presence of 5α-cholest-9(11)-en-23-one-3β,6α,20*S*-triol 3-sulfate (Thornasterol A) as the aglycone in **1**.[12]

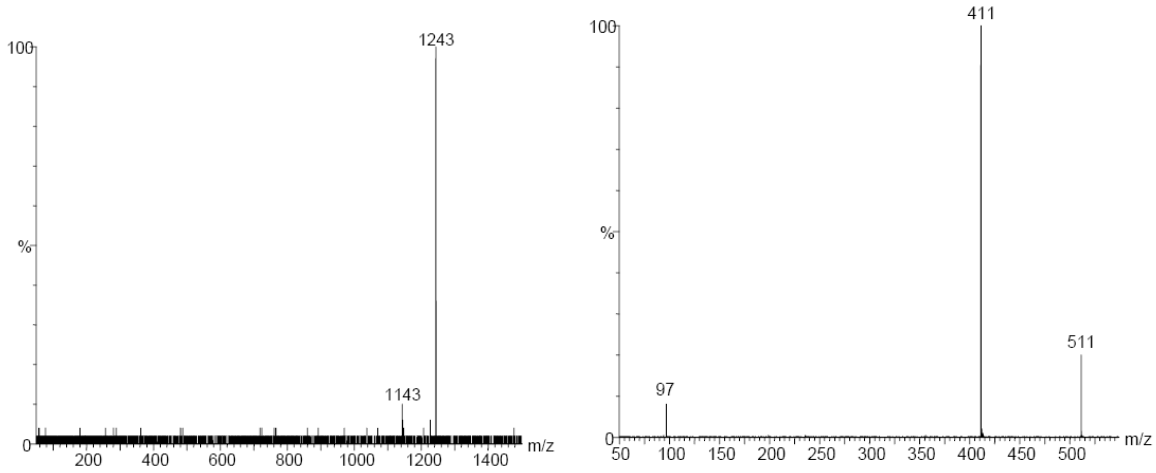

Figure 3. (a) ESI-MS/MS of the ion of *m/z* 1243; (b) ESI-MS/MS of the ion of *m/z* 511.

The ESI-MS of fraction F4 (Fig. 2) detects ions arising from the cleavages of the glycosidic bonds from the terminal sugar moieties of asterosaponin **1**, with charge located on the aglycone containing fragments. A fragment ion of *m/z* 1097 ($[M - 146 - Na]^-$) indicates loss of a terminal deoxyhexose residue (attributable to isomeric fucose or quinovose) whereas the fragment ions of *m/z* 951 ($[M -146 -146 - Na]^-$) and 935 ($[M - 146 - 162 - Na]^-$) are consistent with the loss of terminal quinovose and fucose units and fucose and galactose, respectively. Figure 2 also shows ions of *m/z* 999 and 837 due to the retro aldol cleavage of the aglycone side chain ($C_6H_{12}O$ loss) from fragment ions of *m/z* 1097 and 935, respectively.

The ion of *m/z* 495 in Fig. 2 could not be assigned to fragmentation of Thornasteroside A (**1**). ESI-MS/MS of this ion shows SO_4H^- of *m/z* 97 as the only fragmentation product. The fragment ion of *m/z* 495 has been detected as a characteristic ion in the FAB mass spectrum in negative ion mode of asterosaponins containing (20*R*)-5α-cholest-9(11)-en-23-one-3β,6α-diol 3-sulfate as the aglycone. ^{13}C-NMR analysis of fraction F4 shows the presence of signals at δ 11.9 (C-18) and 210.4 (C-23) ppm characteristic of this aglycone.[3] This data is consistent with the presence of

several saponin spots in the TLC of fraction F4 and the detection of ions of *m/z* 1431, 1407, 1391, 1257 and 1229 in addition to the desodiated anion (*m/z* 1243) of Thornasteroside A (**1**) (Fig. 2).

Fraction F8 obtained by purification of the *n*-BuOH extract of the central disks of *H. helianthus* contained sulfated polyhydroxysteroid glycosides as the major components. The ESI-MS of F8 in the negative ion mode (Fig. 4) displays an abundant ion of *m/z* 663 corresponding to the desodiated anions of isomeric steroidal glycosides **2** and **3**, together with ions of *m/z* 677, 795 and 807, presumably due to desodiated anions of minor polyhydroxylated steroidal glycosides. To investigate their structures, ESI-MS/MS experiments were performed.

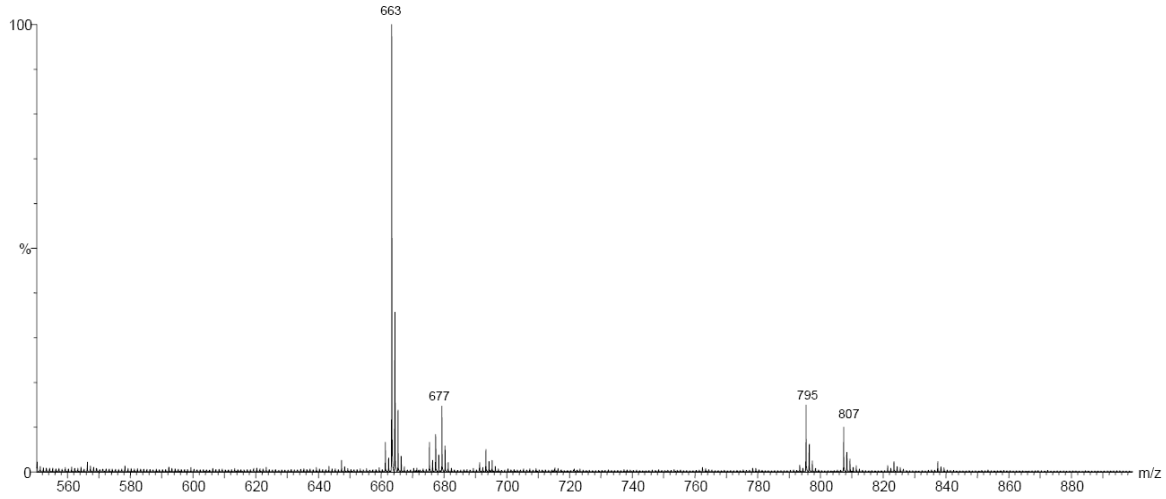

Figure 4. ESI-MS in the negative ion mode of the mixture of polyhydroxylated glycosides in fraction F8.

The ESI-MS/MS of the isolated ion of *m/z* 663 (Fig. 5) shows the SO_4H^- of *m/z* 97 as the major fragment together with a fragment ion of *m/z* 211 assigned to the sulphated xylose unit in Helianthoside (**2**).

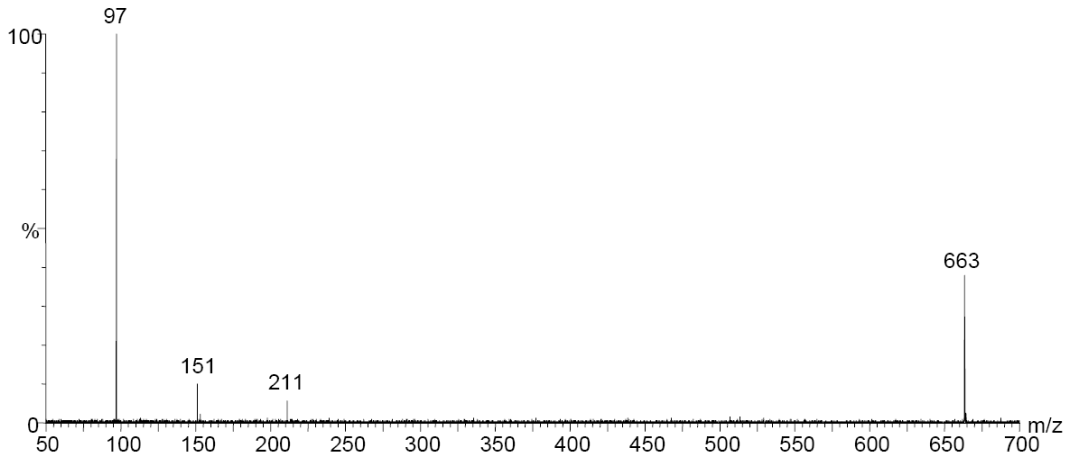

Figure 5. ESI-MS/MS of the ion of *m/z* 663.

^{1}H-NMR analysis of fraction F8 shows diagnostic signals that confirm the presence of glycosides **2** and **3** in this fraction (Table 1).[5]

Table 1. Selected ^{1}H NMR signals for Helianthoside (**2**) and its isomer (**3**) in fraction F8

Proton	**2**	**3**
	δ (m, J Hz)	δ (m, J Hz)
H_3-18	1.30 (s)	0.95 (s)
H_3-19	1.23 (s)	1.01 (s)
H_3-21	1.00 (d, 6.0)	0.94 (d, 7.0)
H_3-26	1.08 (d, 6.8)	0.89 (d, 6.7)
H_3-27	1.13 (d, 6.8)	0.88 (d, 6.7)

ESI-MS/MS experiment of the ion of *m/z* 677 ion (Fig. 6a) shows it to dissociate mainly to HSO_4^- of *m/z* 97 as well as to a fragment ion of *m/z* 529 by the loss of a pentose unit via H transfer. This fragmentation pathway indicates that the ion of *m/z* 677 corresponded to a sulfated monoglycoside of a polyhydroxylated steroid 14 Da heavier than Helianthoside (**2**) and its isomer (**3**). This mass difference can be attributed to the presence of a methoxy group in one of the sugar residues. This structural feature is very common in polyhydroxysteroid glycosides isolated from starfishes. On the other hand, the ESI-MS/MS of the isolated ion of *m/z* 807 (Fig. 6b) shows it to dissociate to an ion of *m/z* 657 by the loss of one pentose unit via H transfer as well as to a fragment ion of *m/z* 211 as observed in the ESI-MS/MS of the ion of *m/z* 663. This results, together with the detection of HSO_4^- as the major fragment, are consistent with a sulfated polyhydroxysteroid diglycoside detected as $[M - Na]^-$ of *m/z* 807.

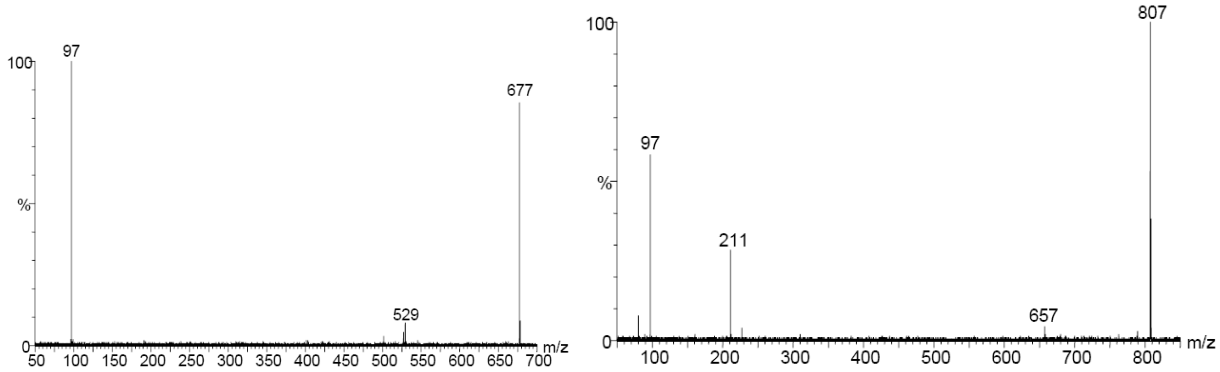

Figure 6. (a) ESI-MS/MS spectrum of ion at *m/z* 677; (b) ESI-MS/MS spectrum of ion at *m/z* 807.

Subsequent ESI-MS/MS of the ion of *m/z* 795 shows it to dissociate mainly to HSO_4^- and a fragment ion of *m/z* 211 presumably due to a sulphated pentose unit as in Helianthoside (**2**). The mass difference between monoglycosides **2** and **3** and the glycoside of *m/z* 795 is 132 Da and corresponds to the mass of a pentose. Hence, the ion of *m/z* 795 can be assigned to a sulfated polyhydroxysteroid diglycoside containing two pentose units, one bearing a sulfate group.

Conclusions

Electrospray ionization mass and tandem mass spectrometries in the negative ion mode is shown to provide a fast and suitable screening method able to characterize underivatized sulfated steroidal glycosides in purified extracts of starfish. The present study indicates that fractions F4 and F8 contain complex mixtures of asterosaponins and sulfated mono- and diglycosides of polyhydroxylated steroids, respectively. ESI-MS and ESI-MS/MS together with NMR data allowed us to confirm the presence of saponins **1**-**3** in and detect the presence of other minor sulfated steroidal glycosides that may contribute to the toxicity of the starfish.

ESI-MS and ESI-MS/MS have great advantages for chemical screening of complex saponin mixtures. Due to the time-consuming and material-consuming isolation of pure saponins, these methodologies are important tools to obtain information on the complexity of a saponin mixture, the presence of known compounds and the chance of finding new structures. However, because the information is limited on the sugar linkage pattern, site of attachment of sulfate groups and the presence of sugar isomers, only a partial characterization of a new saponin is possible and further studies on the pure compounds using NMR spectroscopy and chemical reactions are required.

Experimental Section

General Procedures. ^{1}H and ^{13}C NMR spectra were recorded in Py-d_5 on a Bruker AM 500 spectrometer. TLC was performed on precoated Si gel F254 (*n*-BuOH-HOAc-H_2O (12:3:5)) and C_{18} reversed-phase plates (70% MeOH-H_2O) and detected by spraying with *p*-anisaldehyde (5% EtOH). Samples were analyzed by direct infusion ESI by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μl/ min. Negative ion mode ESI-MS fingerprints and negative mode ESI-MS/MS for low energy collision-induced dissociation (CID) were acquired using a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer. Capillary and cone voltages were set to 3300 V and 30 V respectively, with a desolvation temperature of 100 °C. Each sample was prepared in a solution containing 50% (v/v) chromatographic grade methanol (Tedia, Fairfield, OH, USA) and 50% (v/v) deionized water basified with ammonium hydroxide (Merck, Darmstadt, Germany). Mass spectra were acquired in the range between *m*/z 100 and 1400. ESI-MS/MS was performed by selecting the ion of interest using the first quadrupole Q1, which was in turn subjected to 15–35 eV collisions with argon in the second rf-only collision quadrupole (Q2) while scanning the orthogonal TOF mass analyser to acquire its tandem mass spectrum.
Specimens of *H. helianthus* were collected at Las Cruces (V Región, Chile). The organisms were identified by Dr. Claudia Muniain of the Museo de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina, where a voucher specimen is preserved (MACN-IN 36525).

Extraction and isolation of steroidal glycosides. The starfish (10 animals, 3.85 kg) were defrosted and dissected into their arms (2.7 kg) and central disks (1.14 kg). Each portion was cut into small pieces, homogenized in EtOH and filtered. The EtOH was evaporated to obtain an aqueous residue that was partitioned between H_2O and cyclohexane and then between *n*-BuOH and H_2O. The *n*-BuOH extracts were evaporated under reduced pressure to give glassy materials (9.5 g from the arms and 3.5 g from the central disks) toxic to the brine shrimp *Artemia salina* (LD_{50}: 107.3 ppm and 264.3 ppm, respectively). Each *n*-BuOH extract was chromatographed on a Sephadex LH60 column with MeOH:H_2O (2:1) as eluent to give fractions containing mixtures of sulfated saponins. Two of these fractions, F4 (99 mg) and F8 (139 mg) were selected for ESI mass spectrometry studies.

Acknowledgements

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