Extraction, identification and biological activities of saponins in sea cucumber *Pearsonothuria graeffei*

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Abstract

Aim and objective: Secondary metabolism in marine organisms produced a diversity of biological important natural compounds which are unpresented in terrestrial species. Sea cucumbers belong to the invertebrate Echinodermata and are famous for their nutraceutical, medical and food values. They are known for possession triterpenoid glycosides (saponins) with various ecological roles. The current work aimed to separate, identify and test various biological activities (anti-bacterial, antifungal, antileishmanial and anticancer properties) of saponins produced by the holothurian *Pearsonothuria graeffei* from the Red Sea, Egypt.
Material and Method: The structures were identified by 1D and 2D NMR (^{1}H, ^{13}C, TOCSY, COSY, HSQC, HMBC, and ROESY) experiments and acid hydrolysis. The crude and purified fractions was analyzed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)/MS to identify saponins and characterize their molecular structures. Partially purified fraction containing mainly compounds **1** and **2** was screened for antifungal activity against three clinical isolates of *Candida albicans* (*Candida* 580 (1), *Candida* 581(2) and *Candida* MEO47228. Antileishmanial activity against *Leishmania major* and toxicity on colon cell-line were also evaluated.
Results: Two lanostane type sulfated triterpene monoglycosides were isolated from the Holothurian *Pearsonothuria graeffei* from the Red Sea, Egypt. Holothurin A (**1**) and echinoside A (**2**) triterpene saponins were separated by reversed phase semi-preparative HPLC. LC_{50} values (µg/mL); calculated for the fraction containing

saponins **1** and **2** as major constituents; against *Candida albicans*, *Leishmania major* and colon cell-line were 10, 20 and 0.50, respectively.
Conclusion: Consequently, this study demonstrated the potential use of sea cucumber *Pearsonothuria graeffei* not only as appreciated functional food or nutraceuticals but also as the source of functional ingredients for pharmaceutical products with antifungal, antileishmanial and anticancer properties

Keywords: *Pearsonothuria graeffei*, Holothuria, Sea cucumber, Saponins, triterpene glycosides, Antifungal, Antileishmanial, Anticancer.

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1. INTRODUCTION

Holothurians are commonly known as sea cucumbers belonging to invertebrate echinoderms and has been exploited for their nutritional and medical values. Sea cucumbers have been used as a food source, especially for South East Asia [1] and their market demand is increasing as they are also recognized as a source of bioactive compounds [2,3]. The extracts from sea cucumbers have been shown to have curative properties for weakness, bronchial inflammation and arthritis [4-9].

Because of the diversity of marine organisms and habitats, secondary metabolism in marine organisms has produced a number of very important compounds [10,11]. So far, numerous thousand (26,000) marine metabolites have been defined from marine microbes, seaweeds, polychaetes, soft corals, sponges, bryozoan, ascidians and other pelagic and benthic organisms [12,13,14]. Representatives of all the main chemical classes of secondary metabolites have been found in marine invertebrates and algae including alkaloids, peptides, terpenoids, shikimates, phenols, steroids, polyketides, resins and saponins [15,16]. The secondary produced natural products are ecologically important and provide multiple ecological roles for marine organisms and communities [17-23].

Saponins (soap-like compounds) which are derivatives from triterpenoids consider the main compounds that seem to defend sea cucumbers chemically [24,25]. The identity and toxicity of saponins produced by sea cucumbers varies from species to species [26]. They have been studied from sea cucumbers for more than sixty years. In the past 25-30 years, information on structural diversity, taxonomic distribution, biological activities and biological roles of these substances has been reviewed [27-30]. The number of triterpene glycosides isolated from

holothurians over the past four decades represents 700 compounds with an average of 15 isolated compounds each year [31].

Triterpene glycosides (saponins) are natural products of mixed biogenesis, composed of a carbohydrate moiety and a triterpene aglycone. To the present time natural products of this chemical class were found from animals only in holothuroids, asteroids and recently in sponges and corals [32, 33]. Chemically, triterpene saponins of sea cucumbers differ significantly from those of terrestrial plants [34, 35]. Some of their biological activities including haemolysis, ichthyotoxicity are much higher in comparison with those of terrestrial saponins [35]. The majority of these glycosides are remarkable derivatives of the tetracyclic triterpene lanosterol (1) and have a skeleton of a hypothetical lanostan-3-β-ol-(18-20)-lactone (2) named as holostanol [36,37]. In addition to the various biological properties, triterpene glycosides (saponins) are of great importance to holothurians during maturation and ovulation regulation processes [38-39].

In the course of our continuous interest in identifying bioactive compounds from marine organisms [3,40,41], we have investigated secondary metabolites produced by Red Sea Holothurian (*Pearsontrhuria graeffei*) to look for and identify the bioactive compounds especially saponins which may be of biomedical use thereby raising the importance of sea cucumbers as a biometrically useful species which should be conserved.

2. MATERIALS AND METHODS

2.1. General experimental procedures

The spectra of the 1D and 2D NMR were obtained on the Varian VNMRS 500 spectrometer operating at 125 MHz for 13 C and 500 MHz for 1 H. All chemical shifts for NMR spectra were referenced to the internal standard DMSO at δ_H 2.50 and δ_C 39.52. To determine precisely the molecular masses of the active ingredients in the crude and purified extracts of the sea

cucumbers, the MALDI-TOF mass spectrometry was used here as an accurate and rapid method. MALDI-TOF MS method was carried out using a Bruker Reflex IV MALDI-TOF Mass spectrometer with a 337 nm nitrogen laser was used and was operated in reflection mode. The spectra were processed and analyzed using Bruker flex Control software. For a typical MALDI-TOF analysis, the samples (about 10 µL) and matrix solutions (about 50µL) were added and mixed in a 0.2 ml Eppendorf tube. Upon application of the sample (1 µl) to the support, it was dried on the 384 position micro-titre format sample plate. Thin layer chromatography was conducted on silica gel F_{245} plates (Merck, Darmstadt, Germany) and detected by spraying with 10 % H_2SO_4 followed by heating. The eluent system $CHCl_3$/MeOH/AcOH/H_2O 15:8:3:2 was used for saponin separations; $CHCl_3$/MeOH 9:1 for sapogenins and $CHCl_3$/MeOH/H2O 8:5:1for monosaccharides. The Komarowsky reagent, 2% 4-hydroxybenzaldehyde in MeOH/ 50 % H_2SO_4 soln. 5 :1 was used as sprayer for saponins and diphenylamine/phosphoric acid reagent for the sugars. HPLC analysis was carried out with a Varian RI detector using the YMC ODS-A column (10 × 250 mm, 5µm) and the Varian polaris NH_2 column (4.6 × 250 mm, S-5µm). All of the used reagents, solvents and sugar standards were of high quality grade (Fluka and Sigma).

2.2. Sample materials

Samples of *Holothurian* (*Pearsonothuria graeffei*) were collected from the Gulf of Aqaba, Red Sea and kept frozen until used. Identification of *Holothurian Pearsonothuria graeffei* was kindly accomplished by Dr Mohamed Ismail (Suez Canal University, Ismailia, Egypt.). A specimen voucher was deposited at the Museum of Marine Science Department, Suez Canal University.

2.3. Extraction and isolation

Before extraction, the *Pearsonothuria graeffei* body wall tissues were break into small pieces and lyophilized, then soaked overnight 4 times into adequate quantities of methylene chloride / methanol (1:1, v/v). The combined extracts were dried at 40 ^{o}C in a rotary evaporator. Evaporated extracts were dissolved in water and sequentially partitioned between *n*-hexane and *n*-butanol.

2.3.1 Partial Purification of crude extract

2.3.1.1 Solid phase extraction

In this study, solid phase extraction technique was used here as a powerful, rapid and cost effective technique to purify and fractionate the crude extract. Silica gel (C_{18}) (Waters) Sep-Pak cartridges were primarily conditioned with methanol (3 m) followed by distilled water (5 ml). After that, the butanol extract containing 150 mg was loaded through the C_{18} cartridge. Elution of the cartridge started with 10 mL of distilled water and then sequentially eluted with 10 mL volumes of 20%, 40%, 60%, 80% and 100% v/v of methanol in water to fractionate the crude extract to various fractions. Ideally the carbohydrates pass readily through a C_{18} Sep-Pak with water, and most of the flavonoids and phenolic acids can be removed with 40 % methanol at which concentration the majority of saponins were retained on the solid phase. Activity of the selected fractions was limited to the 80% and 100% methanol fractions.

2.3.2 Isolation

The 85% methanol fraction (4.0 g) was apportioned over reversed silica gel (ODS flash column), gradually eluted with 500 ml of MeOH / H_2O (50, 60, 70, 80, 90 and 100) to yield 6 fractions (A - F). Based on bioactivity assays and ^{1}H NMR monitoring, fractions C (700 mg) and E (400 mg) were assigned for additional chromatography. HPLC (ODS) was used to purify these

fractions using solvent system of 70 % MeOH / H_2O to provide two compounds, holothurin A (**1**) and echinoside A (**2**) were isolated as pure compounds.

2.4 Acid hydrolysis and sugar analysis

The isolated saponin solutions (5.0 mg/ 2 mL H_2O) were added to 2.0 N CF_3COOH (5 mL) and refluxed for 6 h at 75 °C. Mixed reactants were diluted with 10 mL H_2O and extracted three times with 20 mL EtOAc, then washed with H_2O and dried to obtain the aglycon. The aqueous layer was repeatedly evaporated to dryness with MeOH and the residue was dissolved in CH_3CN-H_2O (1:1) and then analysed by TLC (*c*) and HPLC. HPLC analysis performed by using an isocratic elution of CH_3CN-H_2O (90:10) with RI detection in a flow rate 0.9 mL / min. Co-elution experiments with standard sugar samples allowed the identification of quinovose (t_R = 9.97 min), methyl glucose (t_R = 10.54 min), xylose (t_R = 11.17 min), and glucose (t_R = 19.74 min). Co-injection of each hydrolysate with standard D-quinovose, D- methyl glucose, D-xylose, and D- glucose gave consistent peaks. The obtained elutes were collected individually, dried and finally dissolved in water, then their optical rotation was measured.

2.5 Bioactivity testing

Efficiency of the purified fractions were tested on clinical and laboratory isolates of the bacteria *Pseudomonas aeroginosa*, *Staphylococcus aureus*, and *Escherichia coli* and on the fungal pathogen *Candida albicans*. Isolates were obtained from Hull Royal Infirmary (Clinical Microbiology Services), UK. Tests were carried out in 96-well microtitre plates [42]. In short, the extracts were diluted in dimethyl sulfoxide (DMSO) to different concentrations (Extract dry weight). The crude and purified extracts were then incubated with actively growing cells in the appropriate fresh medium. DMSO only was used as control for bioassays. We used four replicates for each data point. Microbial growth was evaluated after 24 hours. Extracts were

considered inactive if optical density of fungal or bacterial cultures was more than 90% of control cultures after 24 hours.

The MTT Assay was used here to determine anti-leishmania activity and to assess cell proliferation. In this assay, the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is reduced ant anti-proliferative activity was measured. This technique is for cells in a 96 well plate as mentioned above except that cells were incubated at 24 °C. Following 20 hours incubation, 50 µl of MTT (5.0 mg/ml) in PBS solution was added to each well, then plates were incubated for another 4 hours. The plates were centrifuged at 750 g for 10 min and pellets were re-suspended in DMSO before adding Sorenson buffer. The plate contents were mixed and absorption was measured at 570 nm.

The colorectal adenocarcinoma cell line (LoVo) (kindly provided by Dr. J. Greeman, Biomedical Research Laboratory, Hull University) was used for assessing anti-proliferative activity. The assay was implemented as for leishmania except that incubations were performed at 37 ◦C. Cell lines were cultured in DMEM medium (Invitrogen, Paisley, United Kingdom) supplemented with 10% (v/v) heat inactivated fetal calf serum (Invitrogen) , 2 mM L-glutamine and 100 U/mL penicillin-streptomycin mixtures in a humidified atmosphere of 5% CO_2 and 95% O_2. Proliferative activity was assessed using MTT reduction after 24 h. colorectal cell lines were preserved in liquid nitrogen.

3. RESULTS AND DISCUSSION

3.1 Structure elucidation of the isolated saponins

The obtained MeOH/CH_2CL_2 extract of sea cucumber *Pearsonothuria graeffei* was partitioned sequentially between *n*-hexane, *n*-butanol and H_2O. The butanol fraction was re-

dissolved in 85 % MeOH and partitioned with *n*-hexane. The flash chromatography followed by semi-preparative ODS-HPLC were used to purify the methanolic fraction. As result, two triterpene saponins, holothurin A (**1**) [Figure 1] and echinoside A (**2**) [Figure 2] were isolated. Structural elucidation of the saponins was mainly determined by 1D and 2D NMR experiments (^{1}H, ^{13}C, DEPT, COSY, TOCSY, ROESY, HSQC, and HMBC) and MALDI-TOF. The correlation of the designated COSY and HMBC of compound **2** is shown in Figure 3 which confirmed the identity of compound 2.

Holothurin A (**1**) was isolated as a white amorphous powder. Based on a combination of a peak at m/z 1244.23 $[M+Na]^{+}$ of MALDI-TOF MS and the ^{13}C NMR spectrum, its molecular formula was established as $C_{54}H_{85}NaO_{27}S$. Structure was also established as 3-*O*-{(3-*O*-methyl-*β*-D-glucopyranosyl)- (1→3)-*β*-D-glucopyranosyl)- (1→4)- *β*-D-quinopyranosyl)- (1→2)- 4-O-sodiumsulfate-*β*-D-xylopyranosyl}22,25 epoxyholosta-9(11)- ene- 3β,12α,17α-triol based on the complete assignment of all the ^{1}H and ^{13}C-NMR signals (Table 1 and 2) by 2D NMR analysis linked to sugar chain and the aglycon moiety. This saponin was the first time reported from the sea cucumber *Holothuria leucospilota Brandt* [43].

Echinoside A (**2**) was isolated as a white amorphous powder. Its molecular formula was established as $C_{54}H_{87}NaO_{26}S$ on the basis of a combination of a peak at m/z 1230.29 $[M+Na]^{+}$ of MALDI-TOF MS and the ^{13}C NMR spectrum. The TOCSY, COSY, HMBC and HSQC experiments (Table 1 and 2) resulted in the full assignment of all NMR signals correlated with the glycosidic chain and aglycon moiety and were well-matched with previous data of the echinoside identified from the sea cucumbers *Holothuria scabra* for the first time and also from *Actinopyga echiinites* [44]. Thus, the structure of **2** was established as 3-*O*-{(3-*O*-methyl-*β*-D-

glucopyranosyl)- (1→3)-*β*-D-glucopyranosyl)- (1→4)- *β*-D-quinopyranosyl)-(1→2)-4-O-sodiumsulfate-*β*-D-xylopyranosyl}holosta-9(11)-ene- 3β,12α,17α-triol .

3.2 Biological activities

Over the range of 6-100 µg/mL, none of the tested crude or partially purified extracts showed any useful activity against the two pathogenic bacterial species (*Pseudomonas aeroginosa* and *E. coli*).

3.2.1 Antifungal activity

The data presented in Figure 4 show the effects of the crude and purified extracts of the sea cucumber *Pearsonothuria graeffei* on the pathogenic fungal species *Candida albicans*. It was found that the purified fraction had good antifungal activity with different sensitivities. The calculated 24 hrs LC_{50} was found to be 10 µg/mL. These results, therefore, confirmed the antifungal activity determined in the primary assays by enriching the activity and reducing the LC_{50} values.

3.2.2 Anti-Leishmanial Activity Testing

The crude and purified extracts of the sea cucumber *Pearsonothuria graeffei* were tested for the anti-leishmanial activities. The purified extracts showed good activity towards *Leishmania major* in the range of 15 to 20 µg/ mL (Figure 4). Furthermore, the ranges of MIC_s on Leishmania were higher than those determined on *Candida* across the range of tested concentrations (5 – 100 µg/ mL).

3.2.3 Anti-proliferative activity

Both of the crude and purified extracts of the sea cucumbers *Pearsonothuria graeffei* were also tested against the proliferative activities of the colon cell-lines. Figure 5 shows that the purified extracts showed very good activity with LC_{50} below 1.0 µg/mL over the range of

concentration (0.15 - 5 µg/mL). The anti-proliferative activities were found to be enriched by the purification process and reduced three times more than the crude extracts (Figure 5). This also indicated that the anti-proliferative activities are related to the mixture of purified saponins present in these organisms. Interestingly the high biological activities detected in the sea cucumber *Pearsonothuria graeffei* could be correlated to habitat where it inhabit the reef flat characterized with high intensity of predation as a result of variety of coral reef fishes and other macroconsumers. There is considerable information on differences in production of chemical defences and potential effects on ecological and evolutionary patterns and processes [45-47]. Typically the activity against cancer cell lines was greater than that observed for either the yeast or Leishmania assays. This high anticancer activity also corroborates with most of the recent publications [48,49], which link these activities to the presence of saponins. Saponins (oligoglycosides) have been found exclusively in echinoderms within the animal kingdom. Potent antiproliferative activity was lately assigned against a number of cell lines including colon, breast and others [50-55].

The high level of activity against cell lines and the overall anti-eukaryotic activity observed in this work and those of previous authors allow us to propose that the biological activity of these secondary metabolites is possibly related to defence against predation by fish or other higher organisms. It might also reduce fouling and pathogenicity caused by other microorganisms living in or attaching to the surface of the Holothurian *Pearsonothuria graeffei*. One suggestion was that saponins are important in reproduction, anti-predation, and anti-fouling [56-59]. In fact the variability in secondary metabolite expression needs not to be related to patterns in anti-consumer defence alone. This is because secondary metabolites can serve a

multiple functions to sea cucumbers, including: prey defences, antifouling, antimicrobial activity and pathogenic parasites.

4. CONCLUSION

The saponins **1** and **2** were extracted and characterized using different techniques. LC_{50} values (µg/mL); calculated for the fraction containing saponins **1** and **2** as major constituents; against *Candida albicans*, *Leishmania major* and colon cell-line were 10, 20 and 0.50, respectively. Consequently, this study demonstrated the potential use of sea cucumber *Pearsonothuria graeffei* not only as appreciated functional food or nutraceuticals but also as the source of functional ingredients for pharmaceutical products with antifungal, antileishmanial and anticancer properties. The identified two saponins in this study may be used for antifungal, antileishmanial and anticancer treatments corresponding to the potent activity detected in partially purified fractions. Another possible use of these two saponins may be acid hydrolysing and production of aglycones**.** These steroidal cores can be used as an intermediate in chemical synthesis and production of steroids.

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Table 1. ^{1}H and ^{13}C NMR Data of the Aglycon Portion of Compounds 1 and 2 (DMSO, δ in ppm).

position	δ_H (mult, *J* in Hz) **1**	δ^{13}C	δ_H (mult, *J* in Hz) **2**	δ^{13}C
1a 1b	1.۳۷ (1 H, *m*) 1.۷1 (1 H, *m*)	35.7 (CH_2)	1.35 (1 H, *m*) 1.69 (1 H, *m*)	35.7 (CH_2)
2a 2b	1.64 (1 H, *m*) 1.84 (1 H, *m*)	26.3 (CH_2)	1.64 (1 H, *m*) 1.83 (1 H, *m*)	26.3 (CH_2)
3	3.01 (1 H, *m*)	88.0 (CH)	3.00 (1 H, *m*)	88.0 (CH)
4		39.0 (C)		39.3 (C)
5	0.84 (1 H, d, J = 10.6)	52.0 (CH)	0.84 (1 H, d, J = 10.2)	52.0 (CH)
6a 6b	1.44 (1 H, m) 1.63 (1 H, m)	20.5 (CH_2)	1.46 (1 H, m) 1.64 (1 H, m)	20.5 (CH_2)
7a 7b	1.65 (1 H, m) 1.80 (1 H, m)	27.5 (CH_2)	1.31 (1 H, m) 1.67 (1 H, m)	27.3 (CH_2)
8	2.87(1 H, m)	39.6 (CH)	2.87(1 H, m)	39.9 (CH)
9		152.7 (C)		152.7 (C)
10		38.8 (C)		38.9 (C)
11	5.24 (1 H, d, J = 4.2)	114.5 (CH)	5.24 (1 H, br d, J = 4.4)	114.6 (CH)
12	4.40 (1 H, d, J = 4.7)	70.2 (CH)	4.43 (1 H, d, J = 4.7)	70.2 (CH)
13		57.5 (C)		57.5 (C)
14		45.0 (C)		45.4 (C)
15a 15b	1.65 (1 H, m) 1.72 (1 H, m)	35.9 (CH_2)	1.01 (1 H, m) 1.69 (1 H, m)	35.9 (CH_2)
16	1.93 (1 H, m) 1.95 (1 H, m)	34.5 (CH_2)	1.93 (1 H, m) 2.39 (1 H, m)	34.8 (CH_2)
17		88.5 (C)		88.3 (C)
18		173.5(C)		173.8 (C)
19	1.03 (3H, s)	22.0 (CH_3)	1.03 (3H, s)	22.0 (CH_3)
20		86.1 (C)		86.4 (C)
21	1.36 (3H, s)	18.5 (CH_3)	1.42 (3H, s)	22.5 (CH_3)
22	4.10 (1 H, m)	79.5 (CH)	1.63 (1 H, m) 2.54 (1 H, ddd, J = 4.1)	37.9 (CH_2)
23a 23b	1.89 (1 H, m) 1.98 (1 H, ddd, J = 4.1)	27.1 (CH_2)	1.29 (1 H, m) 1.39 (1 H, m)	21.4 (CH_2)
24a 24b	1.62 (1 H, m) 1.71 (1 H, ddd, J = 4.1)	37.8 (CH_2)	1.16 (1 H, m) 1.39 (1 H, m)	39.2 (CH)
25		80.9 (C)	1.53 (H, m)	27.3 (CH)
26	1.20 (3H, s)	28.5 (CH_3)	0.86 (3H, s)	22.6 (CH_3)
27	1.15 (3H, s)	27.1 (CH_3)	0.85 (3H, s)	22.5 (CH_3)
30	0.80 (3H, s)	16.2 (CH_3)	0.81 (3H, s)	16.2 (CH_3)

31	1.00 (3H, s)	27.5 (CH_3)	1.00 (3H, s)	27.5 (CH_3)
32	1.18 (3H, s)	19.5 (CH_3)	1.19 (3H, s)	19.3 (CH_3)

Table 2. ^{1}H and ^{13}C NMR Data of the Sugar Portion of Compounds 1 and 2 (DMSO, δ in ppm).

Position	δ_H (mult, *J* in Hz) **1**	δ ^{13}C	δ_H (mult, *J* in Hz) **2**	δ ^{13}C
Xyl				
1	4.33(1H, *d*, *J* = 7.0)	104.0 (CH)	4.33(1H, *d*, *J* = 7.0)	104.0 (CH)
2	3.36 (1H, *m*)	81.5 (CH)	3.35 (1H, *m*)	81.6 (CH)
3	3.52 (1H, *m*)	74.4(CH)	3.51 (1H, *m*)	74.5(CH)
4	3.94 (1H, *m*)	74.3 (CH)	3.93 (1H, *m*)	74.4 (CH)
5a	3.21 (1H, *m*)	63.0 (CH_2)	3.20 (1H, *m*)	63.1 (CH_2)
5b	3.95 (1H, *dd, J = 4.7, 12.0)*		3.94 (1H, *dd, J = 4.7, 12.0)*	
Qui				
1	4.49 (1H, *d*, *J* = 7.8)	103.6 (CH)	4.49 (1H, *d*, *J* = 7.8)	103.7 (CH)
2	3.05 (1H, *m*)	74.9 (CH)	3.05 (1H, *m*)	74.9 (CH)
3	3.29 (1H, *m*)	74.3 (CH)	3.29 (1H, *m*)	74.3 (CH)
4	3.00 (1H, *m*)	86.0 (CH)	2.99 (1H, *m*)	86.2 (CH)
5	3.32 (1H, *m*)	70.2 (CH)	3.32 (1H, *m*)	70.2 (CH)
6	1.22 (3H, d, *J* = 6.1)	17.5 (CH_3)	1.24 (3H, d, *J* = 6.1)	17.4 (CH_3)
Glc				
1	4.38 (1H, *d*, *J* = 8.1)	103.0 (CH)	4.39 (1H, *d*, *J* = 8.1)	103.0 (CH)
2	3.20 (1H, *m*)	72.2 (CH)	3.20 (1H, *m*)	72.2 (CH)
3	3.39 (1H, *m*)	87.9 (CH)	3.37 (1H, *m*)	87.6 (CH)
4	3.21 (1H, *m*)	68.5 (CH)	3.21 (1H, *m*)	68.5 (CH)
5	3.30 (1H, *m*)	76.5 (CH)	3.30 (1H, *m*)	76.2 (CH)
6a	3.35 (1H, *m*)	61.0 (CH_2)	3.38 (1H, *m*)	60.9 (CH_2)
6b	3.65 (1H, *m*)		3.68 (1H, *m*)	
Me Glc				
1	4.35 (1H, *d*, *J* = 7.6)	103.8 (CH)	4.36 (1H, *d*, *J* = 7.6)	103.9 (CH)
2	3.15 (1H, *m*)	73.6 (CH)	3.14 (1H, *m*)	73.6 (CH)
3	2.96 (1H, *m*)	86.0 (CH)	2.98 (1H, *m*)	86.0 (CH)
4	3.14 (1H, *m*)	69.3 (CH)	3.13 (1H, *m*)	69.3 (CH)
5	3.22 (1H, *m*)	76.8 (CH)	3.21 (1H, *m*)	76.8 (CH)
6a	3.40 (1H, *m*)	60.5 (CH_2)	3.40 (1H, *m*)	60.8 (CH_2)
6b	3.70 (1H, *m*)		3.68 (1H, *m*)	
3- MeO	3.49 (3H, *s*)	60.1	3.48 (3H, *s*)	60.0

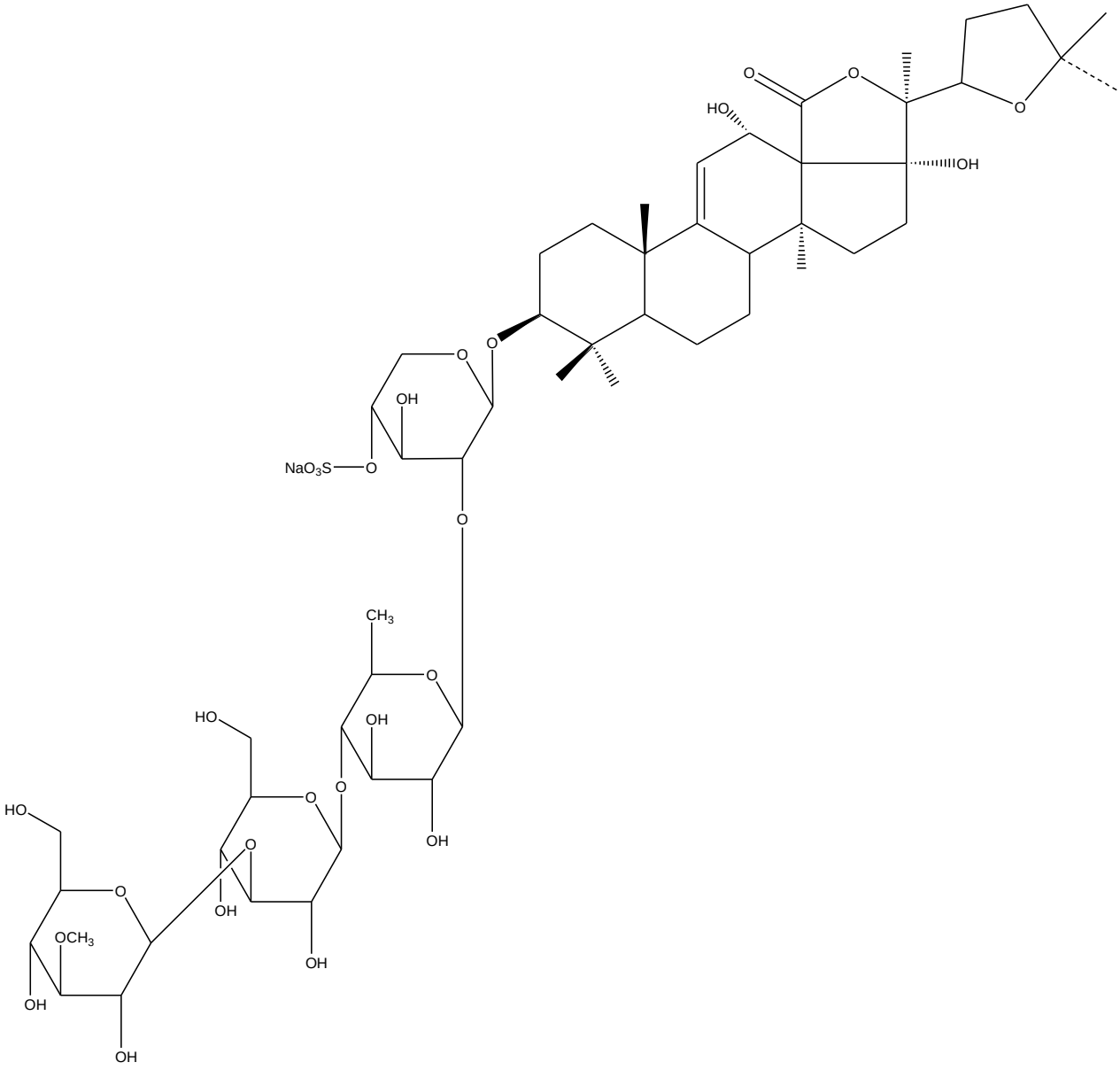

Figure1 : Chemical structure of holothurin A (compound 1).

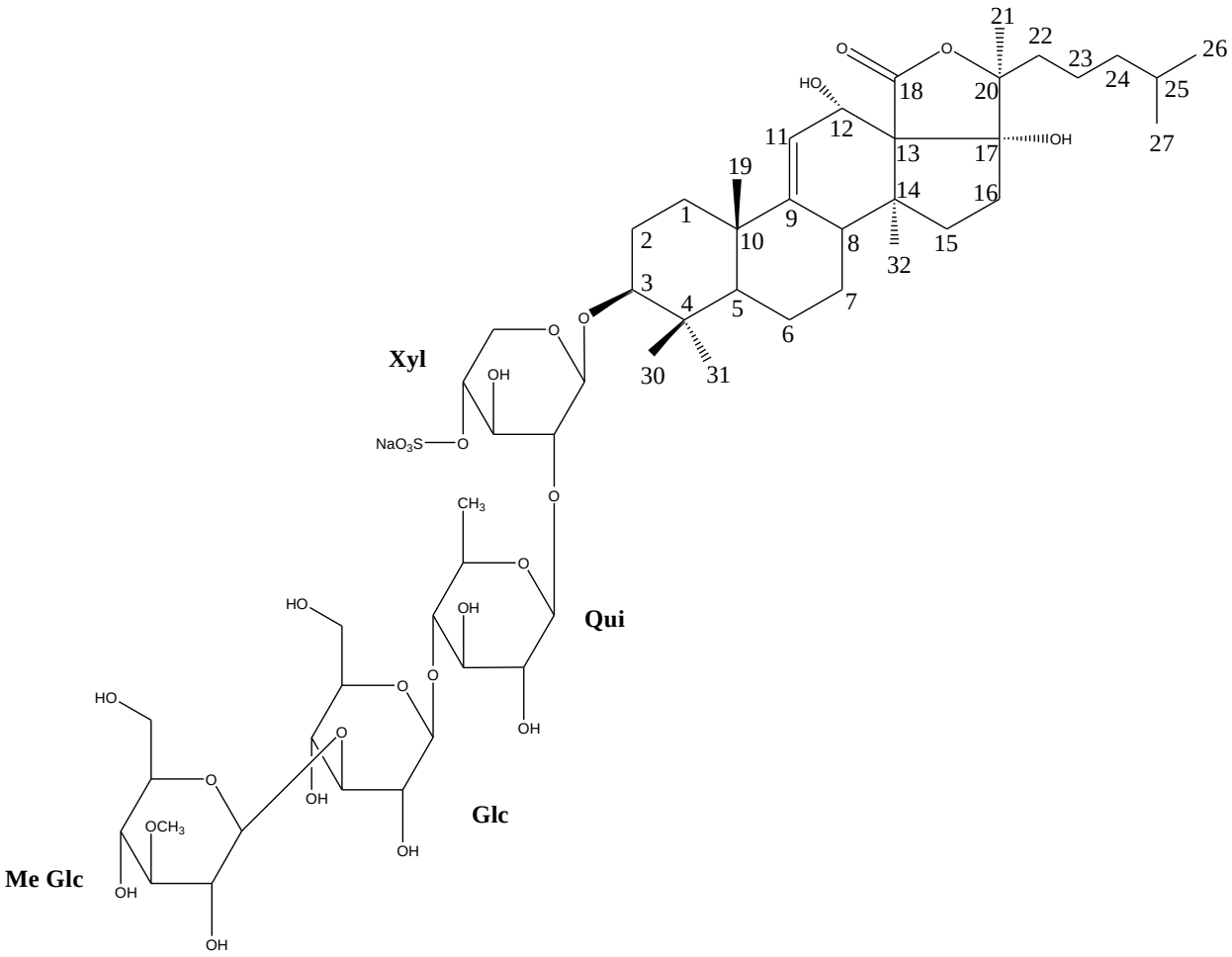

Figure2 : Chemical structure of echinoside A (compound 2).

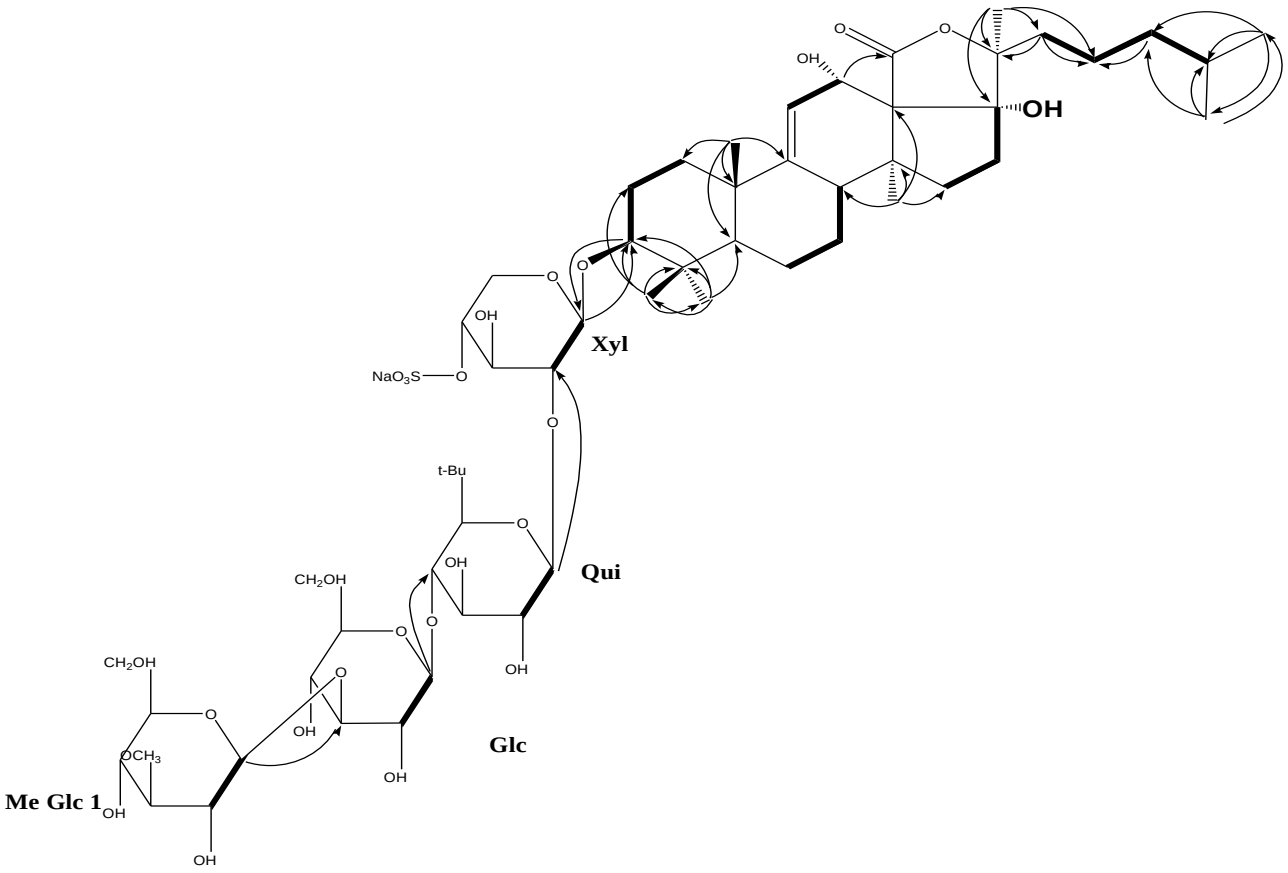

Figure3: Selected HMBC and COSY correlations of compound 2.

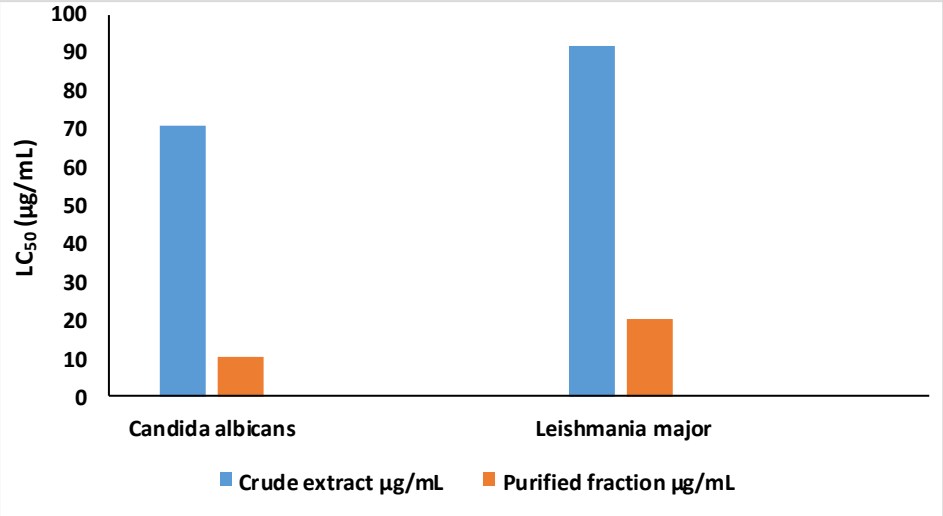

Figure 4. 24 hrs LC_{50} values (µg/mL) calculated for crude and purified extracts of the sea cucumbers *Pearsonuthuria graeffei* against *Candida albicans* and *Leishmania major*.

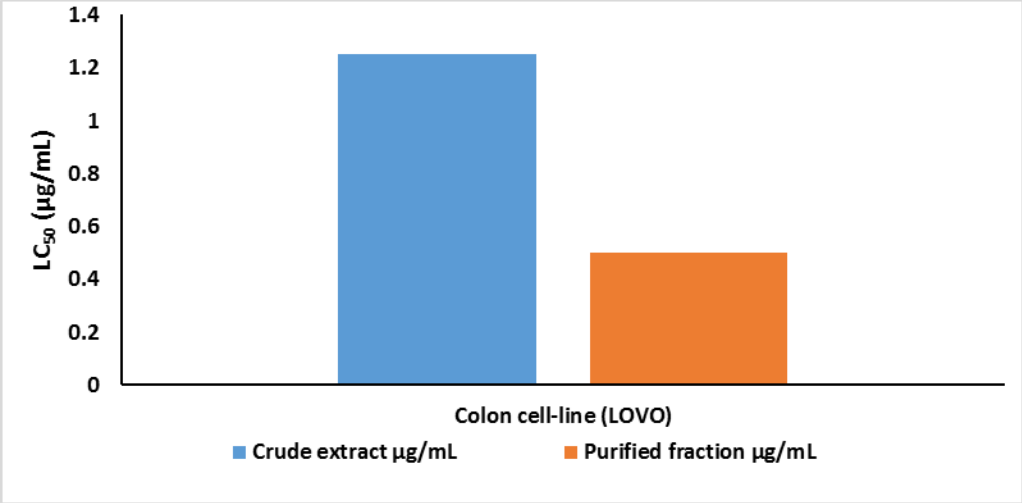

Figure 5. 24 hrs LC_{50} values (µg/mL) calculated for crude and purified extracts of the sea cucumbers *Pearsonuthuria graeffei* against colon cell-line.