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Original Article

Phytochemical analysis of *Holothuria leucospilota*, a sea cucumber from Persian Gulf

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Abstract

Sea cucumbers are widely consumed in traditional medicine and food. These animals have a considerable secondary metabolite and also several potential biological activities. This study investigated the phytochemical and cytotoxic evaluation of *Holothuria leucospilota (H. leucospilota)*, a sea cucumber from Persian Gulf. The saponin composition of *H. leucospilota* was studied by different partitioning and chromatography methods such as thin layer chromatography (TLC), medium pressure liquid chromatography (MPLC) and high performance LC (HPLC). The marine sea cucumber Holostane-type triterpenoids (1-3) were characterized by physical and spectroscopic examination (1 and 2 dimensional neuclear magnetic resonance and mass experiments) with data analysis. The structure of compounds **1-3** identified as echinoside A, holothurine A, and 24-dehydroechinoside A, showed moderate cytotoxic activity with IC_{50} values of 1.9 ± 0.07, 6.8 ± 0.23, and 2.57 ± 0.18 µg/mL against HeLa and 10.4 ± 0.32, 8.9 ± 0.24, and 4.4 ± 0.13 on HUVEC cell line, respectively. In conclusion, the holostane-type triterpenoids showed moderate cytotoxic activity against HeLa cell line and have a prosperous future to be introduced as a lead structure.

Keywords: *Holothuria*; Persian Gulf; Saponin; Sea cucumber.

INTRODUCTION

Marine environment is an incomparable source of secondary metabolites, with structures, which have not typically found in terrestrial plant natural products. In the last decade, wide range of new structures isolated from marine habitat points that marine organisms render a great number of novel bioactive structures (1,2). On the other hand, marine animals are a prime source of potent secondary metabolites with pharmaceutical activities compared to microorganisms, marine plants, terrestrial plants and animals (3). Therefore, the marine invertebrates are an appropriate source of highly-occurrence cytotoxic compounds (4).

Sea cucumbers (*Holothuridae* and *Stichopodidae*) have been utilized in Chinese or oriental medicine and food stuffs (5) and also commercially from past to present (6). Sea cucumbers specially *Holothuridae* is a rich sources of chondroitin sulfates, triterpen saponin glycosides, sulfated polysaccharides, glycosaminoglycan, cerberosides, phenolics, sterols (glycosides and sulfates), lectins, glycoprotein, glycosphingolipids, peptides, and essential fatty acids. In Chinese and Malaysian literature sea cucumber has been studied for their capability against hypertension, rheumatism, asthma, impotence and constipation (5,7-9). These organisms indicate a variety of potent pharmacological activities such as antiangiogenic, antitumor, anticoagulant, antimicrobe, anti-cancer, antioxidant, and anti-inflammatory, and antithrombotic activities (10-13).

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Moreover, holothurians are rich in vitamins and metals, but they are also consumed because they are believed to be an aphrodisiac food. Sea cucumbers are a rich source of saponins, and in particular triterpen glycosides, composed of an aglycone based on the lanostane skeleton and an oligosaccharide chain, with a variable number of monosaccharaides (from 1 to 6), one or more of them bearing a sulfate group (14). The biological activity of saponins is generally attributed to their strong membranolityc action following their interaction with sterols of cellular membrane (15). Saponins from holothurians seem to have an ecological role as defense compounds against predators (16), although they can also have a significant function in attracting symbionts (17).

Sea cucumber *Holothuria leucospilota (H. leucospilota)* is an edible sea cucumber with a high content of bioactive compounds. To date, some papers have been showing the potential biological activities of extracts of *H. leucospilota*; however, only a few studies on the chemical profile, and more specifically on the saponin profile of *H. leucospilota* have been performed, and they are limited to specimens collected near the south China Sea (18,19)

The aim of this study was phytochemical analysis of *H. leucospilota*, a sea cucumber from Persian Gulf and evaluation of their cytotoxic activity against HeLa and HUVEC (normal) cell lines.

MATERIALS AND METHODS

General experiment and procedures

Column chromatography (CC), lichroperp RP-18 25-40 µm; thin layer chromatography (TLC), silica gel GF254 plates (20 × 20 cm, 0.5 mm (Merck, Germany); screening by spattering with cerium sulphate (Sigma, USA) in 10% aq. H_2SO_4 and using heat. All solvents used were of high pressure liquid chromatography (HPLC) grade (Caledon Laboratory Chemicals Ltd. Georgetown, Ont. Canada). Neuclear magnetic resonance (NMR; Bruker, USA) AV-400 (1H) and AV-100 (13C); EI-MS: waters Q-TOF Micro YA019 mass spectrometer (USA).

Animal material

Specimens of the sea cucumber *H. leucospilota* were collected by scuba diving from the coast of Persian Gulf, Bushehr, I.R. Iran. The animal was frozen immediately.

Extraction and isolation

The frozen specimens of *H. leucospilota* were freeze dried and minced. The powder (220 g) exhaustively extracted five times in 1 L EtOAc: methanol (1:1). The crude extract (35 g) was partitioned successively through Kupchan partitioning method to hexane, dichloromethane, BuOH and water (17). The BuOH extract (15 g) was fractionated by medium PLC (MPLC) on RP-18 25-40 µm) eluted with H_2O: MeOH (0-100%, stepwise) to give 80 fractions. All the fractions were checked with TLC and NMR for their saponin contents. Some fractions containing saponins were finally purified to yield compounds **1-3**. The final purification of compounds **1**, **2**, and **3** was performed on an HPLC column Luna C-18 (250 mm × 4.60 mm) with gradient elution with H2O (A) and MeOH (B), 50-55% B over 25 min, 55-100% B over 10 min holding for 10 min and flow rate 0.8 mL/min (tR^{1st}, 15.56 min; tR^{5th}, 10.6 min; and tR^{6th}, 16.2 min).

Cytotoxicity evaluation

The epitheloid cervix carcinoma cell line, HeLa, and human umbilical vein endothelial cells, HUVEC were provided by the Pasteur Institute of Iran, Tehran, I.R. Iran. Cells were incubated in a humidified incubator with 5% CO2 at 37 ºC. HeLa and HUVEC cells were fed with Roswell Park Memorial Institute (RPMI) medium and Dulbecco's Modified Eagle's medium (DMEM), respectively. The media were fed with 10% (v/v) fetal bovine serum (FBS) and 100 IU/mL penicillin or streptomycin.

To identify bioactive compounds from the algae, cytotoxicity of four different partitions including water, butanol, dichloromethane, and hexane were firstly evaluated, and then the most effective partition was subjected to further fractionation using HPLC. Twelve fractions were also screened for their cytotoxic effects. *In vitro* cytotoxicity of samples against HeLa as well as HUVEC cells

was evaluated using 3-(4, 5-dimethyl – 2 - thiazolyl)-2, 5 – diphenyl - 2H - tetrazolium bromide (MTT) assay as explained previously. Briefly, a cell suspension of 2×10^5 cells/mL was seeded in 96-well plates and incubated for 24 h for cell attachments. The dried fractions or partitions were dissolved in dimethyl sulfoxide (DMSO) (the final DMSO concentration in the palate was less than 1%) and 20 µL of different concentrations (0.5-4 µg/mL) of each sample was added and incubated at 37 °C for 72 h in a humidified atmosphere. Then cells were incubated with 20 µL of MTT solution (5 mg/mL) at 37 °C for 3 h. The medium was removed and MTT-formazan crystals dissolved and 150 µL of DMSO was subjoined and the absorbance was measured at 570 nm by a plate reader. Doxorubicin was used as positive control, and cells treated with DMSO (1%) was used as negative control. The cell survival was calculated according to the following equation:

$$Cell\ survival\ (\%) = \frac{Absorbance\ of\ treated\ well - absorbance\ of\ blank}{Absorbance\ of\ control\ well - absorbance\ of\ blank} \times 100$$

Statistical analyses

All data are expressed as mean ± standard deviation (SD) and the statistical analysis was performed by SPSS version 20 software. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test.

RESULTS

Extraction and isolation

The EtOAc:MeOH (1:1) extract of *H. Leucospilota* (220 g, dried) was partitioned through Kupchan partitioning method to hexane, dichloromethane, n-BuOH, and H_2O. The n-BuOH extract was consecutively fractionated to MPLC on LiChroprep® RP-18 (25-40µm) to yield the pure echinoside A (**1**), holothurine A (**2**) and 24-dehydroechinoside A (**3**). These three known compounds were isolated and identified by comparison of 1 and 2 D-NMR and mass experiments with data analysis and previous literatures.

Structural determination of Echinoside A

Echinoside A (1): colorless crystals; infrared (IR) ν max 3380 (hydroxyl group), 1745(γ-lactone group), 1260 and 827 (sulfate group), and 1070 cm^{-1}; ^1H and ^{13}C-NMR data, see Table 1; ESI-MS m/z 1208.3096 $[M - Na]^+$; (calcd for $C_{54}H_{87}NaO_{26}S$, 1207.3096).

Echinoside A (**1**) was a crystal with molecular formula of $C_{54}H_{87}NaO_{26}S$ by the molecular ion [M-Na] at m/z 1207.30 in the ESI-MS (positive-ion mode). The IR spectrum showed an absorption band at 1745 cm^{-1}, strong broad absorption bands at 3380 and 1070 cm^{-1} as well as two further absorption bands at 1260 and 1210 cm^{-1}. The ^1H-NMR, ^{13}C-NMR, and heteronuclear multiple bond correlation (HMBC) spectra (Tables 1 and 2) showed resonances related to seven CH_3 groups at δH 0.8, 0.84, 0.86, 0.99, 1.02, 1.20, 1.41 (δC 16.1, 22.4, 22.4, 27.4, 21.9, 19.3, 22.5), one alkenyl group (δH 5.23; δC 114.5, 152.6) and one lactone carbonyl group (δC 174.0). The spectra also showed a broad singlet resonance due to a CH proton linked to a carbon with a hydroxyl group at δH 4.42 (δC 70.1) and a quaternary oxygenated carbon at δ 87.9,

The HMBC experiment determined all proton connectivity with those of their corresponding carbons that are separated by two, three, and, sometimes in conjugated systems, four bonds. Through HMBC experiment, the signals of interglycosidic linkages, relative to shifts expected for the corresponding methyl glycopyranosides, are downfielded; δC 81.4 (xylose C-2, Xyl), δC 86.2 (quinose C-4), δC 87.6 (glucose C-3) (19). A cross-peak between the H-1 of Xyl and C-3 of the aglycone showed the connection of Xyl and C-3 of the aglycone, then the interglycosidic linkages could be derived the same way. The location of linkage of the sulfate group in the sugar units was determined by studying the NMR chemical shifts of the sugar moiety, C-4 (Xyl) shifted downfield to 74.3 ppm, explaining that the sulfate group was located at C-4 of the Xyl unit. The structure of the carbohydrate chain of compound **1** was corroborated by HMBC correlations which has already been confirmed by previous researches (19).

Structural determination of Holothurin A

Holothurins A (2): colorless oil; IR (neat) ν max 3420 (hydroxyl group), 1745 (γ-lactone), 1640 (diene bond), 1225 and 827 (sulfate group) and 1070 cm^{-1} (oxido moiety); ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS m/z 1222.2931 in a positive ion mode ($C_{54}H_{85}NaO_{27}S$ $[M + 1]^{+}$); (calc for $C_{54}H_{85}NaO_{27}S$ 1221.2931 *m/z*).

The structure, carbohydrate moieties, and its ^{13}C-NMR spectral data of glycosides of compound **2** are shown in Table 1 and Fig. 1. Same as compound **1,** these carbohydrate chains contain 3-O-methyl-glucopyranosyl, glucopyranosyl, quinovopyranosyl, and xylopyranosyl residues containing a sulfate group correlated with C-4 of the Xyl unit. The molecular formula of compound **2** was determined as $C_{54}H_{87}O_{26}SNa$ by a pseudomolecular ion $[M+Na]^{+}$ at m/z 1243.3689 EI-Mass (positive ion mode), calculated for $C_{54}H_{85}NaO_{27}S$, 1221.2931 m/z (20).

Table 1. 1H and 13C-NMR data (400 MHz basic frequency, CDCl3) for Echinoside A (**1**), Holothurin A (**2**) and 24-dehydroechinoside A (**3**) (400 MHz, CDCl3).

Position	Compounds					
	1		2		3	
	δH	δC	δH	δC	δH	δC
1a	1.03, 1H	35.6	1.84 m	36.2	1.03, 1H	36.4
1b	1.35, 1H		1.40 m		1.35, 1H	
2a	1.64, 1H	26.2	1.93 m	26.8	1.64, 1H	27.1
2b	1.84, 1H		2.11 m		1.84, 1H	
3	3.03, 1H	87.8	3.16 dd	88.5	3.03, 1H	88.6
4		39.2		39.8		40.0
5	0.85, 1H	51.9	1.02 m	52.5	0.85, 1H	52.7
6a	1.44, 1H	20.2	1.56 m	21.0	1.44, 1H	21.2
6b	1.63, 1H		1.77 m		1.63, 1H	
7	1.33, 1H	27.3	1.78 m	28.2	1.33, 1H	28.3
7b	1.66, 1H				1.66, 1H	
8	2.86dd,1H	39.8	3.42 m	40.7	2.86 dd,1H	40.8
9		152.6		153.4		154.2
10		39.0		39.5		39.6
11	5.23d, 1H	114.5	5.65 dd	115.3	5.23 d, 1H	115.6
12	4.42 brs, 1H	70.1	5.05 d	71.3	4.42 brs, 1H	71.2
13		58.5		59.0		58.5
14		45.4		45.8		46.2
15a	1.01, 1H	35.8	1.49 m	36.7	1.01, 1H	27.1
15b	1.70, 1H		1.87 m		1.70, 1H	
16a	1.93, 1H	34.8	2.52 m	35.4	1.93, 1H	35.8
16b	2.38, 1H		3.45 m		2.38, 1H	
17		87.9		89.9		89.1
18		174.0		174.5		174.5
19	1.02 s, 3H	21.9	1.40 s	22.3	1.02 s, 3H	20.0
20		86.3		87.2*d*		86.8
21	1.41 s, 3H	22.5	1.90 s	19.3	1.41 s, 3H	22.9
22a	1.74, 1H	37.8	4.18 dd	80.7	1.74, 1H	36.6
22b	1.84, 1H				1.84, 1H	
23	1.34, 2H	21.3	1.86 m, 1.93 m	30.7	6.13 dt	120.6
24	1.15, 2H	38.6	1.37 m, 1.84 m	36.2*c*	6.03 d	143.6
25	1.53, 1H	27.0		81.3	1.53, 1H	28.3
26	0.86d, 3H	22.4	0.89 s	22.7	0.86 d, 3H	22.6
27	0.84d, 3H	22.4	0.88 s	22.4	0.84 d, 3H	22.7
30	0.80 s, 3H	16.1	1.14 s	16.5	0.80 s, 3H	16.7
31	0.99 s, 3H	27.4	1.29 s	27.9	0.99 s, 3H	27.9
32	1.20 s, 3H	19.3	1.74 s	20.1	1.20s, 3H	22.6

NMR, neuclear magnetic resonance.

Table 2. ^{1}H- and ^{13}C-NMR chemical shifts and selected HMBC correlations for the sugar moiety of isolated compounds (in MeOD, 400 MHz).

Position	Glycosidic parts		HMBC
	δH mult. (*J* in Hz)	**δC**	
1	4.32,1H,MeGlc, (7.6)	104.6	C-3 (Glc)
2	3.13,1H,MeGlc	74.9	
3	2.97, 1H,MeGlc	87.8	
4	3.14, 1H, MeGlc	70.0	
5	3.18, 1H, MeGlc	78.1	
6	3.41, 2H, MeGlc	62.5	
3 OCH3	3.48, 3H, MeGlc	60.4	
1	4.37, 1H, Glc, (7.0)	102.9	C-4 (Qui)
2	3.21, 2H, Glc	72.1	
3	3.37, 2H, Glc	87.6	
4	3.21, 2H, Glc	68.4	
5	3.30, 1H, Glc	76.1	
6a	3.41, 2H, Glc	62.5	
6b	3.68, 2H, Glc		
1	4.34d, 1H, Xyl, (6.4)	103.6	C-3 (Aglycone)
2	3.37, 2H, Xyl	81.4	
3	3.49, 2H, Xyl	74.3	
4	3.49, 2H, Xyl	74.3	
5a	3.20, 1H, Xyl	63	
5b	3.93, 1H, Xyl		
1	4.48d, 1H, Qui, (6.8)	103.9	C-2 (Xyl)
2	3.04, 1H, Qui	74.5	
3	3.28, 1H, Qui	74.8	
4	2.98, 1H, Qui	86.2	
5	3.32, 1H, Qui	70.1	
6	1.23d, 3H, Qui, (4.9)	17.3	C-4, 5 (Qui)

NMR, neuclear magnetic resonance; HMBC, heteronuclear multiple bond correlation; Qui, quinose; Xyl, xylose; Glc, Glucose.

Structural determination of 24 - dehydro-echinoside A

24-dehydroechinoside A (3): colorless crystals; IR ν max 3380(hydroxyl group), 1745(γ-lactone group), 1653 (olefinic group), 1260 and 827 (sulfate group), and 1070 cm^{-1}; ^{1}H and ^{13}C- NMR data, see Table 1; ESI-MS m/z 1206.2937 [M - Na] $^{+}$; (calcd for $C_{54}H_{85}NaO_{26}S$, 1205.2937).

^{13}C-NMR spectral data of the carbohydrate moieties of compound **3** glycosides are shown in Table 1. Similar to compounds **1** and **2,** these carbohydrate chains included 3-O-methyl-glucopyranosyl, glucopyranosyl, quinovopyranosyl, and xylopyranosyl residues with a sulfate group attached to C-4 of the Xyl unit. The basic differences between compounds **1** and **3** was in their side chains; compound **1** possesses a -OH group at C-17 (δC 87.9) while compound **3** has a C=C bond at C-23 δH 6.13 (δC 120.6) and C-24 δH 6.03 (δC 143.6).

Cytotoxicity evaluation

The cytotoxic activity of the pure compounds **1-3** and all partitions against HeLa tumor cell-lines was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The activity was measured based on cell viability after 72 h of treatment. Compounds **1-3** showed cytotoxic activity against the human cervix carcinoma HeLa cell-line, with IC_{50} in the range of 1.9-6.8 µg/mL (Table 3). Among them, compound **1** was the most potent, with IC_{50} of 1.9 ± 0.23 µg/mL. Compounds **1-3** displayed significant cytotoxicity on the growth of HeLa cell line in comparison with positive control.

DISCUSSION

The IR spectrum showed an absorption band at 1736 cm^{-1} (related to a γ-lactone group), strong broad absorption bands at 3424 and 1073 cm^{-1} (related to glycosidic structure) as well as two further absorption bands at 1258 and 1210 cm^{-1} (related to sulphate group).

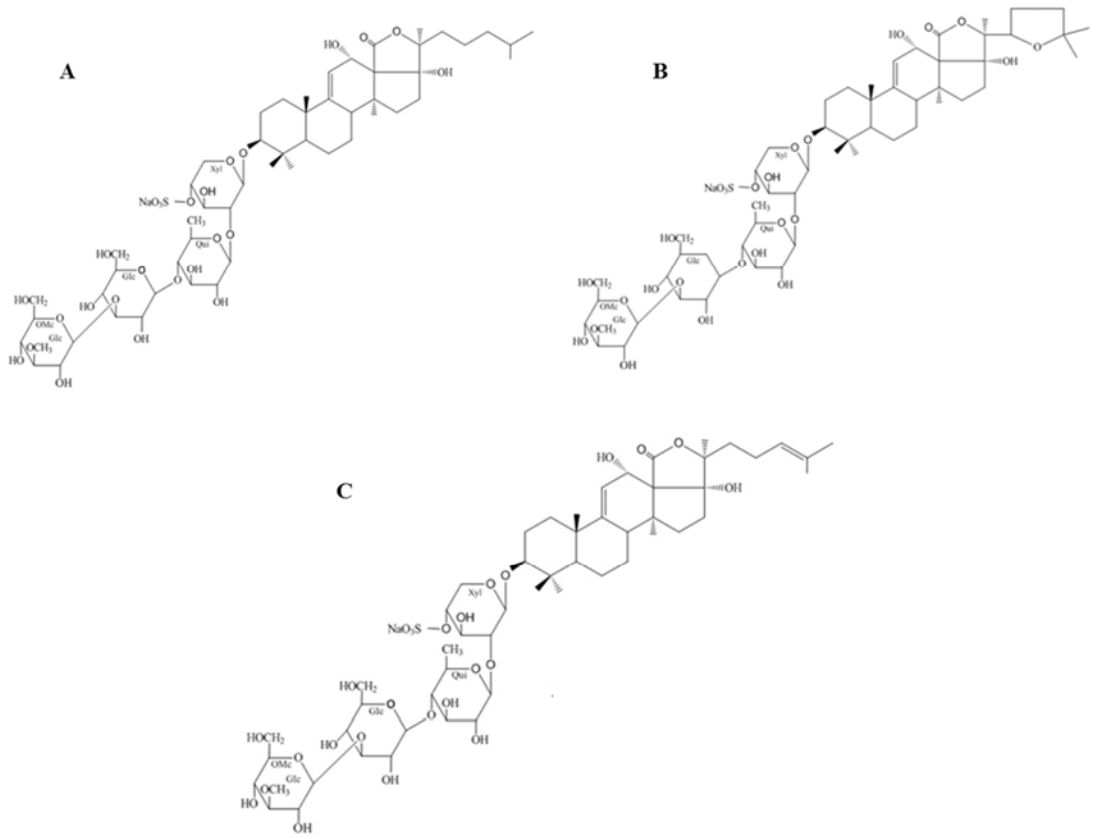

Fig. 1. Chemical structure of triterpene glycosides (A) echinoside A (1), (B) Holothurin A, and (C) 24-dehydroechinoside A.

Table 3. IC_{50} values (µg/mL) of isolated compounds and partitions against Hela and HUVEC cells. Data represent mean ± standard deviation. * Indicates significant differences ($P < 0.05$) in comparison with the positive control.

Compounds or partitions	Hela cells	HUVEC cells
Compound **1**	1.9 ± 0.07*	10.4 ± 0.32
Compound **2**	6.8 ± 0.23*	8.9 ± 0.24
Compound **3**	2.57 ± 0.18*	4.4 ± 0.13
Hexane partition	301 ± 11	-
Dichloromethane partition	210 ± 24	-
Butanol partition	129 ± 12	-
Water partition	229 ± 32	-
Doxorubicin (positive control)	0.6 ± 0.1	

The ^{1}H-NMR, ^{13}C-NMR, and HMBC spectra (Table 1) showed resonances related to seven CH_3 groups at δH 0.8, 0.84, 0.86, 0.99, 1.02, 1.20, 1.41 (δC 16.1, 22.4, 22.4, 27.4, 21.9, 19.3, 22.5), one alkenyl group (δH 5.23; δC 114.5, 152.6) and one lactone carbonyl group (δC 174.0). The spectra also displayed a broad singlet resonance due to a -CH proton linked to a carbon with a -OH group at δH 4.42 (δC 70.1) and a -C- bearing a hydroxyl at δ 87.9, suggesting the presence of the holostane structure with 9 (11) en-3, 12, and 17 triol moiety. The assignments of the NMR signals belonging to aglycone moiety extracted from the H- and C-NMR, led to the identification of aglycone moiety as 9 (11)-holostene-3β, 12α, and 17α triol. The ^{1}H- and ^{13}C-NMR spectra displayed

signals for four anomeric protons and their correlated -C, δH 4.32 (δC 103.9), δH 4.37 (δC 102.9), δH 4.34 (δC 103.6), and δH 4.48 (δC 103.9). It was also exhibited four signals at δ 4.32, 4.34, 4.37, and 4.48, that showed the position of anomeric proton each one doublet with ^{3}J H-1/H-2 = 6.8 to 7.6 Hz, a trans-diaxial orientation according to coupling of β configuration partners represented by the large vicinal coupling constants of each anomeric proton (Tabes 1 and 2).

The tetra saccharide chain assignment, the linkages between the glycosides and the sugar arrangement determined predominantly by HMBC and comparison with reported data (21). Thus, H-3 showed linkage with H-1 (Xyl), H-1α (1.03 ppm), H-5, and H-32 confirming the β configuration at C-3. The coupling constant for H-12 with H-11 (5.23 Hz) was represented the 12α configuration of the -OH group. Final assignments were completed by checking the results of HMBC spectrum (Table 2). The structure of the carbohydrate chain of compound **1** was corroborated by HMBC correlations.

The NMR data represented that compounds **1**-**3** have the same sugar arrangement. Thus, the structure of compound **1** demonstrated to be 3-O-[3-O-methyl-b -D-glucopyranosyl-(1→3)-β -D-glucopyranosyl-(1→4)-b -D-quinovopyranosyl- (1→2)-4-O-sodiumsulfato-β -D-xylopyranosyl]-9(11)-holostene-22-ketone-3β ,12α ,17α -triol. The structure of this compound is shown in Fig. 1.

The molecular formula of compound **2** was identified as $C_{54}H_{87}O_{26}SNa$ by a pseudomolecular ion $[M + Na]^+$ at m/z 1243.3689 EI-mass (positive ion mode), calculated for $C_{54}H_{85}NaO_{27}S$, 1221.2931 m/z. ^{13}C-NMR spectra of glycoside **2** showed the resonance bond of -OH groups at C-12 (71.7 ppm) and C-17 (89.7 ppm) in the holostane structure. The primary signals was confirmed with the corresponding proton signal at 4.95 ppm (H- 12β, J = 5.8 Hz). Moreover, the signal of C-22 and C-25 δH (δC 80.7 and 81.3 ppm, respectively), and one -O-CH_3 (oxomethine) group [δC δC 88.5 (C-3), δH 3.16 (H-3)], demonstrated an oxygen linkage in the side chain position of compound **2**. Correlation from H-21 to C-22, H-23 to C-22 in the HMBC spectrum indicated that carbonyl group belonging to ketone was located at C-22 of the aglycon. These data showed the difference between compounds **1** and **2** in their side chains. Compound **1** has aliphatic side chain and compound **2** has tetrahydrofuran side chain. The ^{13}C-NMR spectrum of the aglycon moiety of compound **2** was identical to that of the holothurin A1 from *H. floridana* (20).

Each of these data including results of 1 and 2D-NMR spectrum was permitted the structural elucidation of this aglycone moiety as 9 (11)-holostene-22-ketone-22, 25 dioxo-3β, 12α, and 17α triol (21). The structure of this compound is shown in Fig. 1.

All the data (Table 1) including results of 2D-NMR experiments allowed identification of this aglycone moiety as 9 (11), 23(24)-dien-holosta-22-ketone- 3β, 12α, and 17α triol (18). The structure of this compound is shown in Fig. 1.

The holostane-type triterpenoids showed moderate cytotoxic activity and have a prosperous future to be introduced as a lead structure. The anti-tumor activity of echinoside A and its structure-activity relationship *in vitro* and *in vivo* have been evaluated. Echinoside A has shown to inhibit activity of cell proliferation by inducing apoptosis in HepG2 cells (24). Furthermore, it is confirmed that the cell cycle could be arrested in the G0/G1 phase with echinoside A. Studies have shown that echinoside A significantly enhanced the expression of the cell-cycle-related genes, such as c-myc, p16, and p21 and reduced the cyclin D1 (25). Nuclear factor kappa B (NF-κB) expression was not affected by echinoside A. This compound with dose of 2.5 mg/kg in mice bearing H22 hepatocarcinoma tumours showed decreased tumor weight by 49.8% (26). According to aforementioned investigations the holostane type compounds, depending on their structure, may induce apoptosis of HepG2 cells in an NF-κB-dependent or NF-κB-independent manner. Zhao *et al*. reported that holothurin A and dehydroechinoside A treatment significantly suppressed adhesion of human hepatocellular

liver carcinoma cells (HepG2) to both matrigel and human endothelial cells (ECV-304) and inhibited HepG2 cell migration and invasion in a dose-dependant manner. Both compounds reduced tube formation of ECV-304 cells on the matrigel *in vitro* and attenuated neovascularization in the chick embryo using the chorioallantoic membrane assay *in vivo*. Immunocytochemistry analyses revealed that these compounds significantly decreased the expression of the matrix metallo-proteinase-9 (MMP-9) and increased the expression level of tissue inhibitor of metalloproteinase-1, an important regulator of MMP-9 activation. Western blot analyses demonstrated that HA (1) and DHEA remarkably abolished the expression of vascular endothelial growth factor (VEGF) (27). Accordingly, holothurin A may activate this pathways on the other cancer cell lines in an NF-κB-dependent or independent manner and with down-regulation of the expression of B-cell lymphoma 2, and increased caspase-3 activation, release of mitochondrial cytochrome c, and poly (adenosine diphosphate ribose) polymerase, cleavage (28,29). In another study, both echinoside A and holothurin A repressed the pancreatic lipase activity and increased fatty acid excretion in the feces. Treatment with both compounds significantly decreased the adipose tissue accumulation in mice. They manifested different inhibitory activities *in vitro*, but each of them dramatically inhibited lipid absorption in vivo and showed strong anti-obesity activity (30). Thus sea cucumbers were shown, once again, to be an important source of bioactive compounds. The analyzed specimen of *H. leucospilota* was shown to contain a number of triterpene glycosides. An unbiased and isolation-independent analytical method like LC-MS/MS combined with molecular networking could be the basis for future, more meaningful comparisons.

CONCLUSION

Holostane-type triterpenoids are the major component of the organic extract of the sea cucumber *H. leucospilota*, collected from the Persian Gulf, and compounds of this type have been shown to have the biological effects for used in pharmaceutical and medical applications. Our studies of *H. leucospilota* have led to the isolation of three known compounds echinoside A, holothurin A, and 24-dehydroechinoside A which exhibited moderate cytotoxic activity against HeLa cell line.

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REFERENCES

1. Sawadogo WR, Boly R, Cerella C, Teiten MH, Dicato M, Diederich M. A survey of marine natural compounds and their derivatives with anti-cancer activity reported in 2012. Molecules. 2015;20(4): 7097-7142.
2. Fenical W, Jensen PR. Developing a new resource for drug discovery: marine actinomycete bacteria. Nat Chem Biol. 2006;2(12):666-673.
3. Munro MH, Blunt JW, Dumdei EJ, Hickford SJ, Lill RE, Li S, *et al*. The discovery and development of marine compounds with pharmaceutical potential. J Biotechnol. 1999;70(1-3):15-25.
4. Faulkner DJ. Marine natural products: metabolites of marine invertebrates. Nat Prod Rep. 1984;1(6):551-598.
5. Bordbar S, Anwar F, Saari N. High-value components and bioactives from sea cucumbers for functional foods-a review. Mar Drugs. 2011;9(10):1761-1805.
6. Zhao YC, Xue CH, Zhang TT, Wang YM. Saponins from sea cucumber and their biological activities. J Agric Food Chem. 2018;66(28):7222-7237.
7. Yaacob HB, Kim KH, Shahimi M, Aziz NS, Sahil SM. Malaysian sea cucumber (Gamat): A prospect in health food and therapeutic. Proceeding of Asian Food Technology Seminar. 1997; p. 6.
8. Zhao Y, Li B, Liu Z, Dong S, Zhao X, Zeng M. Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate. Process Biochem. 2007;42(12):1586-1591.
9. Himaya SW, Ryu B, Qian ZJ, Kim SK. Sea cucumber, *Stichopus japonicus* ethyl acetate

fraction modulates the lipopolysaccharide induced iNOS and COX-2 via MAPK signaling pathway in murine macrophages. Environ Toxicol Pharmacol. 2010;30(1):68-75.
10. Tian F, Zhang X, Tong Y, Yi Y, Zhang S, Li L, *et al*. PE, a new sulfated saponin from sea cucumber, exhibits anti-angiogenic and anti-tumor activities *in vitro* and *in vivo*. Cancer Biol Ther. 2005;4(8):874-882.
11. Wu M, Huang R, Wen D, Gao N, He J, Li Z, *et al*. Structure and effect of sulfated fucose branches on anticoagulant activity of the fucosylated chondroitin sulfate from sea cucumber *Thelenata ananas*. Carbohydr Polym. 2012;87(1):862-868.
12. Ismail H, Lemriss S, Aoun ZB, Mhadhebi L, Dellai A, Kacem Y, *et al*. Antifungal activity of aqueous and methanolic extracts from the Mediterranean sea cucumber, *Holothuria polii*. J Mycol Med. 2008;18(1):23-26.
13. Wijesinghe WA, Jeon YJ, Ramasamy P, Wahid ME, Vairappan CS. Anticancer activity and mediation of apoptosis in human HL-60 leukaemia cells by edible sea cucumber (*Holothuria edulis*) extract. Food Chem. 2013;139(1-4):326-331.
14. Kalinin VI. System-theoretical (Holistic) approach to the modelling of structural-functional relationships of biomolecules and their evolution: an example of triterpene glycosides from sea cucumbers (*Echinodermata, Holothurioidea*). J Theor Biol. 2000;206(1):151-168.
15. Van Dyck S, Caulier G, Todesco M, Gerbaux P, Fournier I, Wisztorski M, *et al*. The triterpene glycosides of *Holothuria forskali*: usefulness and efficiency as a chemical defense mechanism against predatory fish. J Exp Biol. 2011;214:1347-1356.
16. Caulier G, Flammang P, Gerbaux P, Eeckhaut I. When a repellent becomes an attractant: harmful saponins are kairomones attracting the symbiotic Harlequin crab. Sci Rep. 2013;3: 2639-2644.
17. Grauso L, Yegdaneh A, Sharifi M, Mangoni A, Zolfaghari B, Lanzotti V. Molecular networking-based analysis of cytotoxic saponins from sea cucumber *Holothuria atra*. Mar Drugs. 2019;17(2). pii: E86.
18. Van Dyck S, Gerbaux P, Flammang P. Qualitative and quantitative saponin contents in five sea cucumbers from the Indian ocean. Mar Drugs. 2010;8:173-189.
19. Han H, Zhang W, Yi YH, Liu BS, Pan MX, Wang XH. A novel sulphated holostane glycoside from sea cucumber *Holothuria leucospilota*. Chem Biodivers. 2010;7(7):1764-1769.
20. Mamelona J, Pelletier E, Girard-Lalancette K, Legault J, Karboune S, Kermasha S. Quantification of phenolic contents and antioxidant capacity of Atlantic sea cucumber, *Cucumaria frondosa*. Food Chem. 2007;104(3):1040-1047.
21. Li Z, Wang H, Li J, Zhang G, Gao C. Basic and clinical study on the antithrombotic mechanism of glycosaminoglycan extracted from sea cucumber. Chin Med J (Engl). 2000;113(8):706-711.
22. Schillaci D, Cusimano MG, Cunsolo V, Saletti R, Russo D, Vazzana M, *et al*. Immune mediators of sea-cucumber *Holothuria tubulosa* (Echinodermata) as source of novel antimicrobial and anti-staphylococcal biofilm agents. AMB Express. 2013;3(1):35-34.
23. D'Auria MV, Sepe V, D'Orsi R, Bellotta F, Debitus C, Zampella A. Isolation and structural elucidation of callipeltins J-M: antifungal peptides from the marine sponge *Latrunculia sp*. Tetrahedron. 2007;63(1):131-140.
24. Li M, Miao ZH, Chen Z, Chen Q, Gui M, Lin LP, *et al*. Echinoside A, a new marine-derived anticancer saponin, targets topoiso merase2alpha by unique interference with its DNA binding and catalytic cycle. Ann Oncol. 2010;21(3):597-607.
25. Zhao Q, Liu ZD, Xue Y, Wang JF, Li H, Tang QJ, *et al*. Ds-echinoside A, a new triterpene glycoside derived from sea cucumber, exhibits antimetastatic activity via the inhibition of NF-κB-dependent MMP-9 and VEGF expressions. J Zhejiang Univ Sci B. 2011;12(7):534-544.
26. Ye Y, Song Y, Zhuang J, Wang G, Ni J, Xia W. Anticancer effects of echinacoside in hepatocellular carcinoma mouse model and HepG2 cells. J Cell Physiol. 2019;234(2):1880-1888.
27. Zhao Q, Xue Y, Liu ZD, Li H, Wang JF, Li ZJ, *et al*. Differential effects of sulfated triterpene glycosides, holothurin A1, and 24-dehydroechinoside A, on antimetastasic activity via regulation of the MMP-9 signal pathway. J Food Sci. 2010;75(9):H280-H288.
28. Dong L, Debin Yu, Nuoting Wu, Wang H, Niu J, Wang Y, *et al*. Echinacoside Induces Apoptosis in Human SW480 Colorectal Cancer Cells by Induction of Oxidative DNA Damages. Int J Mol Sci. 2015;16(7):14655-14668.
29. Zhao Q, Xue Y, Wang JF, Li H, Long T, Li Z, *et al*. *In vitro* and *in vivo* anti-tumor activities of echinoside A and ds-echinoside A from *Pearsonothuria graeffei*. J Sci Food Agric. 2012;92(4):965-974.
30. Wang Y, Wang J, Yanagita RC, Liu C, Hu X, Dong P, *et al*. Effects of two sulfated triterpene saponins echinoside A and holothurin A on the inhibition of dietary fat absorption and obesity reduction. Biosci Biotechnol Biochem. 2014;78(1):139-146.