A novel antitumor compound nobiliside D isolated from sea cucumber (*Holothuria nobilis* Selenka)

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Abstract. An anticancer compound, triterpene glycoside, was isolated from *Holothuria nobilis* Selenka. Its chemical structure and configuration were determined by two-dimensional nuclear magnetic resonance spectroscopy and electrospray ionization mass spectrometry. The novel active compound was identified as nobiliside D, with the molecular formula $C_{40}H_{61}O_{17}SNa$ and chemical name 3-O-[-β-D-pyranosyl (1-2)-4'-O-sulfon -ate-β-D-xylopyranosyl]-alkoxy-9-ene-3β, 12α, 17α, 25β-4 alcohol. An antitumor test was performed using xCELLigence Real-Time Cell Analysis. Nobiliside D exhibited inhibitory effects on human leukemic cell line K562, human leukemia cell line U937, human lung cancer cell line A-549, human cervix carcinoma cell line HeLa, human breast cancer cell line MCF-7 and human liver carcinoma cell line HepG2. Nobiliside exhibited the greatest inhibitory effect on K562 and MCF-7 cells with an IC_{50} of 0.83±0.14 and 0.82±0.11 μg/ml, respectively. When human tumor cell lines K562 and MCF-7 were treated by nobiliside D (0.5 μg/ml) for 24 h, 45.8% of K562 cells and 58.7% of MCF-7 cells were apoptotic, whereas only 0.5% of un-treated control cells were apoptotic. These data indicate the compound should offer potential as a novel drug for the treatment of a range of cancers.

Introduction

The sea cucumber, *Holothuria nobilis* Selenka *(H. nobilis* Selenka), is a spiny skinned invertebrate with a wide distribution, spreading from the Fujian Dongshan Ocean to the Xisha Islands in China. *H. nobilis* Selenka is rich in biological compounds, including proteins, polysaccharides, vitamins and triterpene glycoside. Of particular interest is triterpene glycoside, the predominant secondary metabolite of the sea cucumber with a range of biological and pharmacological activities, including antitumor, antimicrobial and anti-inflammatory properties (1). For example, impatienside A is a type of triterpene glycoside isolated from the sea cucumber *Holothuria impatiens* (2). Impatienside A has been reported to be effective in the treatment of various tumor cell lines, including HCT-116, A549 and HepG2 (2). The structure of impatienside A is similar to that of bivittoside D and the glycoside shows greater cytotoxicity than other potent anticancer drugs, including etoposide (V-16) (2). Intercedenside D-I, also a cytotoxic triterpene glycoside, is extracted from the sea cucumber *Mensamaria intercedens* and inhibits the proliferation of many human tumor cell lines (3). Another key glycoside is sulfated saponin philinopside, a potential angiogenesis inhibitor isolated from the sea cucumber *Pentacta quandrangulari* that shows anti-angiogenic and antitumor activities (4).

Saponins are a main type of triterpene and are predominantly found in the sea cucumber (5,6). Three novel triterpene glycosides of the saponin category, namely nobilisides A, B and C, were isolated from the sea cucumber *H. nobilis* Selenka. Nobilisides A and C are non-sulfated monoglycosides, while nobiliside B is a sulfated diglycoside. Nobilisides A, B and C possess 22,25-epoxy side chains and nobiliside A contains two conjugated double bonds [22 E, 24-diene and 7,9(11)-diene], a structure seldom found in other glycosides. All three glycosides show substantial cytotoxicity against many tumor cell lines (7).

This cytotoxicity was validated in the present study. Saponins were extracted from the concentrated liquid of *H. nobilis* Selenka and demonstrated inhibitory activity against human leukemic cell line K562, human leukemia cell line U937, human lung cancer cell line A-549, human cervix carcinoma cell line HeLa, human breast cancer cell line MCF-7 and human liver carcinoma cell line HepG2.

Materials and methods

Materials. The human tumor cells lines (K562, U937, A-549, HeLa, MCF-7 and HepG2) were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Gibco™ RPMI-1640 medium was purchased from Thermo Fischer Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Shanghai Lanji Co., Ltd. (Shanghai,

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China). Neutral protease was from Nanning Pangbo Biological Engineering Co., Ltd. (Nanning, China).

H. nobilis Selenka. *H. nobilis* Selenka was collected in June 2012 in Fujian Dongshan Ocean (Fujian, China) and identified by Professor Liao Yulin from Qingdao Ocean University (Qingdao, China). The species was cultured in the Zhejiang Pharmaceutical College (Fuzhou, China).

Saponin extraction from H. nobilis Selenka. Saponin was extracted from *H. nobilis* Selenka and its presence was detected as previously described, with slight modifications (8). *H. nobilis* Selenka (50 g) was washed, chopped and digested with 2% neutral protease (v/v). Insoluble materials were discarded via filtration. The digested solution was added to 30% v/v ethanol made from 95% ethanol at 4˚C for 24 h, then centrifuged at 3,800 x g for 10 min. The supernatants were mixed with 60% ethanol (v/v) of 95% at 4˚C for 24 h and centrifuged at 3,800 x g for 10 min. The supernatant was concentrated to 20% of its original volume through evaporation of water and ethanol, and washed three times with 50 ml diethyl ether (>98%) to remove fat content via a separator funnel. The supernatant was further isolated three times with 150 ml of water-saturated butanol. Alcohol was evaporated and saponins were extracted into n-butanol. The 20 ml n-butanol fraction with saponins was loaded onto a silica gel (200-300 mesh, 0.45 g/ml; Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) column, prior to elution with 1,484 g/ml chloroform, 0.791 g/ml methanol and water (7.5:2.5:1). The eluents (10 ml) were loaded onto a Zobax SB C-18 type ODS reverse phase HPLC column (Agilent Technologies, Inc., Santa Clara, CA, USA; mobile phase composition: 25:75:0.01 acetonitrile/water/acetic acid (v/v/v), at a flow rate of 2 ml/min), and eluted with 80% methanol (v/v) at the flow rate of 1.0 ml/min. Standard sample saponin (catalog no. 47036; Sigma-Aldrich, Merck Millipore, Darmstadt, Germany) was used as an internal control. A purified compound was obtained.

Structure determination. The molecular weight and chemical structure of the final product was identified by gas chromatography-mass spectrometry (GC-MS; Fisons Gas Chromatograph Model GC8000 series 8035 with MD800 Quadrupole mass spectrometer; SpectraLab Scientific, Inc., Ontario, Canada). In the present study, electrospray ionization-mass spectrometry (ESI-MS) of $[M+Na]^+$ions at M/Z 891 and ESI-MS of $[M-Na]^-$ions at M/Z 845 was conducted. The GC column was a AB-35MS fused silica capillary column with dimensions 30x0.25x0.5 mm. The GC conditions were as following: An injection port temperature of 250˚C, an initial column temperature held isothermal at 100˚C before increase to 250˚C at 6˚C/min, then maintained at 250˚C for 10 min. The ion source and interface temperatures were 200 and 250˚C, respectively. Helium gas (180˚C; 7.63 psi) was used as the carrier gas at a speed of 1 ml/min. Spectra were obtained in the electron ionization mode with 70-eV.

The chemical structure of the final product was analyzed by a 600 MHz Varian Inova Nuclear Magnetic Resonance (NMR) spectrometer (Varian, Inc., Palo Alto, CA, USA). The purified saponin was dissolved in 500 ml of 10 mM NaN_3, 1 mM EDTA, 50 mM K_3PO_4, 0.1 M NaCl containing H_2O/D_2O. NMR spectra were compared to deuterated methanol (CD30D; catalog no. 151974; Sigma-Aldrich, Merck Millipore) signals at δ 3.30 (1H) and 49.00 (13C). The samples were dissolved in dimethyl sulfoxide-d6 (DMSO-d6; catalog no. 547239) or deuterated chloroform (CDCl3; catalog no. 441333) (both from Sigma-Aldrich, Merck Millipore) and/or CD3OD, dependent on sample solubility. The observed chemical shift (δ) values are presented as ppm and the coupling constant (J) values in H/Z.

A total of 10 mg purified saponin was mixed with KBr salt. Infrared spectra were analyzed as KBr pellets on a 100 FTIR spectrometer (Thermo Fisher Scientific, Inc.), between 4,000-400 cm^{-1}.

xCELLigence Real-Time Cell Analysis (RTCA). K562, U937, A-549, HeLa, MCF-7 and HepG2 cell lines were cultured in RPMI 1640 medium with 10% FBS at 37˚C and 5% CO_2. Cells were sub-cultured every 2 days and harvested at the exponential growth phase. Cells were then seeded at a concentration of $5x10^4$ cells/well into 100 µl medium and different concentrations of purified saponin (dissolved in 2% DMSO), all within 96-cell micro-plates (#CLS3595; Merck Millipore, Darmstadt, Germany) and incubated for 24 h at 37˚C and 5% CO_2. Different concentrations (20, 30, 40 and 50 mM) of doxorubicin hydrochloride (D4035; Merck Millipore) were used as controls as reported previously (9). Each sample was measured in the E-plate 96 of a xCELLigence-system (catalog no. 05232368001; ACEA Biosciences, Inc., San Diego, CA, USA). Half maximal inhibitory concentration (IC_{50}) values were defined as the inhibition of cell lines by the novel compound. Using the aforementioned method, the inhibitory effects of 0.5 µg/ml purified saponin (the concentration used to measure inhibition), on all cell lines, were measured for 24 h.

Apoptosis assay. The tumor cell lines were cultured at a density of $1x10^5$ cells/ml with 0.5 µg/ml of purified saponin for 24 h. Cells were washed two times with phosphate-buffered saline (PBS) and re-suspended in 250 µl binding buffer (catalog no. 556547; BD Biosciences, San Jose, CA, USA), 5 µl Annexin V-FITC (#A9210) and 10 µl propidium iodide (#81845) (both from Merck Millipore). The mixture was incubated at 25˚C for 5 min in the dark and measured using a fluorescence microscope (#BX-50; Olympus Corporation, Tokyo, Japan). A Dako Cyan flow cytometer (Agilent Technologies, Inc.) was used for quantification.

Statistical analysis. The data from different groups were analyzed using Student's t-test via the SPSS 20.0 software (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Structure determination of purified compounds. Purified saponin was obtained as previously described (10). The chemical characteristics of the purified compound include a solid state of a colorless crystal powder at room temperature, a melting point of 212.4-214.4˚C and positive Liebermann-Burchard and molish reactions. Table I shows the ^{13}CNMR and ^{1}HNMR chemical shifts along with the nuclear overhauser effect

Table I. ^{13}C NMR and ^{1}H NMR chemical shifts and NOESY and HMBC correlations for the aglycon moiety of nobiliside D.

Position	δ_C	δ_H (m, *J* in Hz)	NOESY	HMBC
1	36.4	1.38 (1H, m, α), 1.86 (1H, m, β)		
2	27.4	1.76 (1H, m, α), 2.02 (1H, m, β)	H-3α, H3-30, H3-19	
3	88.8	3.14 (1H, dd, 4.2, 12.0, α)	H-xyl1, H-5α, H3-31	C:xyl$_1$
4	40.1			
5	52.8	1.00 (1H, d, 10.2, α)	H-3α, H3-31	
6	28. 4	2.02 (2H ,m)		
7	21.2	1.49 (1H, m, α), 1.74 (1H, m, β)		
8	41.0	3.36 (1H, t, 9.6, β)	H-7β, H-6β, H-15β, H$_3$-19	
9	153.9			
10	39.8			
11	115.6	5.62 (1H, d, 4.2, β)	H-12β, H-1β, H$_3$-19	C:10,8,13,12
12	71.6	4.99 (1H, dd, 5.4, 12.0, β)	H-11β	C:14,9
13	58.9			
14	46.0			
15	36.9	1.82 (1H, m, α), 1.40(1H, m, β)	H-8β	C:17
16	35.6	2.41 (1H, ddd, α), 2.97 (1H, dd, 6.0, 14.4, β)	H-15α, H$_3$-32 H-15β	C:14,17
17	89.8			
18	174.6			
19	22.7	1.40 (3H, s)	H-1β, H-2β, H3-30, H-8β, H-11β	C:1,10,5,9
20	86.7			
21	18.9	1.76 (3H, s)		C:22,17,20
22	80.7	4.32 (1H, m, α)	H-16β	C:21,20,17
23	28.0	1.45 (2H, m)		C:24,25
24	38.5	1.64 (2H, m)		C:25,27
25	81.4			
26	28.7	1.20 (3H, s)		C:27,25,24
27	27.1	1.19 (3H, s)		C:26,24,25
30	16.8	1.13 (3H, s)	H3-19	C:31,3,4,5
31	28.2	1.28 (3H, s)	H-3α, H-5	C:30,3,4,5
32	20.4	1.65 (3H, s)	H-16α	C:13,14,8,15

NMR conditions, 4:1 pyridine-d_5:D_2O, 600/150MHz; CNMR, carbon-13 nuclear magnetic resonance; HNMR, hydrogen-1 nuclear magnetic resonance; NOESY, nuclear overhauser effect spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy.

spectroscopy (NOESY) and the heteronuclear multiple-bond correlation spectroscopy (HMBC) correlations for the aglycone moiety of purified saponin nobiliside D. Table II shows the ^{13}CNMR and ^{1}HNMR chemical shifts and NOESY and HMBC correlations for the sugar moiety of nobiliside D (in pyridine-d_5:D_2O, 4:1, 600/150 MHz). IR Spectrum analysis demonstrated that the purified saponin exhibited the following characteristics: Max-3418, Strong Broad Peak (Hydroxy), 1762 (Carbonyl), 1637 (Double Bond), 1253, 1069.

The ^{13}CNMR and ^{1}HNMR results indicate that the purified compound is nobiliside D (11), a novel type of saponin with molecular formula $C_{40}H_{61}O_{17}SNa$ and chemical name 3-O-[-β-D-xylopyranosyl(1-2)-4'-O-sulfonate-β-D-xylopyranosyl-alkoxy-9-ene-3β, 12α, 17α, 25β-four alcohol. The structure of nobiliside D is shown in Fig. 1.

Antitumor activity. xCELLigence-system was used to test the cytotoxic effects of nobiliside D on the human tumor cell lines K562, U937, A-549, HeLa, MCF-7 and HepG2, as described previously. As shown in Table III, nobiliside D had strong inhibitory effects on tumor lines K562 and MCF-7, with IC_{50} values of 0.83±0.14 and 0.82±0.11 μg/ml, respectively, although all the inhibitory effects were lower than that of doxorubicin hydrochloride, except for MCF-7.

The growth rate of all cell lines was affected by nobiliside D (Fig. 2). The growth rate of K562, U937 and A-549 cells compared with controls (K562-C, U937-C and A-549-C) was markedly decreased after 1 day of culture (Fig. 2A, $P<0.05$). The growth rate of HeLa, MCF-7 and HepG2 cells compared with controls (HeLa-C, MCF-7-C and HepG2-C) was also markedly different after 1 day of culture (Fig. 2B, $P<0.05$). The

Table II. ^{13}C NMR and ^{1}H NMR chemical shifts and NOESY and HMBC correlations for the sugar moiety of nobiliside D.

Sugar	Position	δ_C	δ_H (m, *J* in Hz)	NOESY	HMBC
Xyl	1	105.3	4.71 (1H, d, 7.2)	H-3, H-3', H-5', H-1''	C-3, C-1''
	2	83.4	4.04 (1H, m)		C-1',C-3',C-1'' H-5'
	3	75.4	4.28 (1H, m)		C-2', C-4'
	4	76.0	5.10 (1H, m)		C-2', C-3'
	5	64.2	3.73 (1H, m, α), 4.75 (1H, dd, 4.8, 11.4, β)	H-1', H-4'	C-4', C-1' C-4'
Xyl (repeat)	1	105.9	4.83 (1H, d, 7.2)	H-3', H-5'', H-2'	C-2'
	2	76.8	4.02 (1H, m)		C-3', C-1''
	3	77.7	4.22 (1H, m)		C-2'
	4	69.9	3.68 (1H, m)		
	5	65.2	3.74 (1H, m, α), 4.55 (1H, dd, 4.2, 10.8, β)		

NMR conditions, 4:1 pyridine-d_5:D_2O, 600/150 MHz; C NMR, carbon-13 nuclear magnetic resonance; HNMR, hydrogen-1 nuclear magnetic resonance; NOESY, nuclear overhauser effect spectroscopy; H MBC, heteronuclear multiple-bond correlation spectroscopy.

Table III. Antitumor activity of nobiliside D on different tumor cell lines.

Tumor cell lines	Nobiliside IC_{50} (μg/ml)	Doxorubicin hydrochloride IC_{50} (μg/ml)
A549	2.43±0.16	1.58±0.45
HeLa	2.90±0.21	1.74±0.16
K562	0.83±0.14	0.01±0.00
MCF-7	0.82±0.11	1.32±0.18
U937	2.97±0.21	0.01±0.02
HepG2	1.43±0.08	0.38±0.04

A-549, human lung cancer cell line; HeLa, human cervix carcinoma cell line; K562, human leukemic cell line; MCF-7, human breast cancer cell line; U937, human leukemia cell line; HepG2, human liver carcinoma cell line; IC_{50}, half maximal inhibitory concentration.

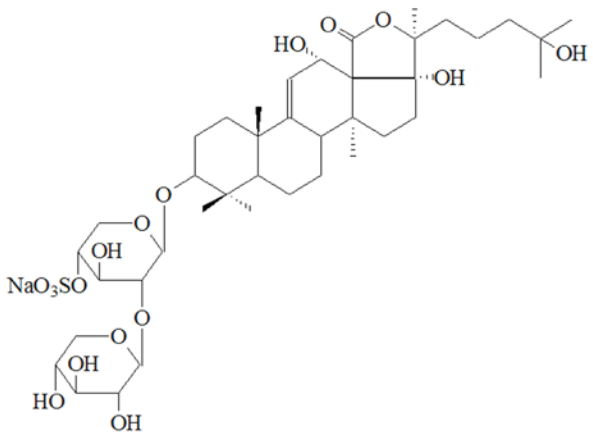

Figure 1. Chemical structure of nobiliside D. Nobiliside D has the molecular formula $C_{40}H_{61}O_{17}SNa$ and the chemical name 3-O-[-β-D-xylopyranosyl (1-2)-4'-O-sulfonate-β-D-xylopyran osyl]- alkoxy-9-ene-3β, 12α, 17α, 25β-four alcohol.

results indicate that nobiliside D has varying inhibitory effects on the different tumor cell lines and that therapy utilizing nobiliside D is potentially most suitable for the treatment of human leukemia and breast cancer.

Nobiliside D promoted apoptosis of human cell lines. A greater apoptotic rate was observed in human tumor cell lines HeLa, A-549, K562, MCF-7, U937 and HepG2 treated by nobiliside D (0.5 μg/ml) for 24 h compared to un-treated cells ($P<0.05$). 18.2% of Hela cells, 16.4% of A-549 cells, 45.8% of K562 cells, 58.7% of MCF-7 cells, 15.6% of U937 cells and 22.4% of HepG2 cells were apoptotic, while only less than 0.5% of untreated control cells were apoptoic (Fig. 3). Additionally, dead cells were identified in all cell line cultures, as previously described (12). The percentages of dead cells were approximately 0.6, 0.9, 2.6. 3.4, 0.7 and 2.0% dead cells for HeLa, A-549, K562, MCF-7, U937 and HepG2 cells, respectively.

Discussion

The present study demonstrated that nobiliside D can be extracted from the sea cucumber *H. nobilis* Selenka. In contrast to the typical polysaccharide extraction protocol using an alcohol precipitation method, extraction of nobiliside D is based on its solubility in specific concentrations of alcohol. Therefore, nobiliside D was extracted from filtrate using 60% ethanol (v/v), enabling precipitation of the compound. The structure of nobiliside D was subsequently identified by NMR spectroscopy and analysis of the spectra of the active fractions. Additionally, the nobiliside D extracted from *H. nobilis* Selenka was identified using ESI-MS and NMR analysis. The novel compound was identified as the

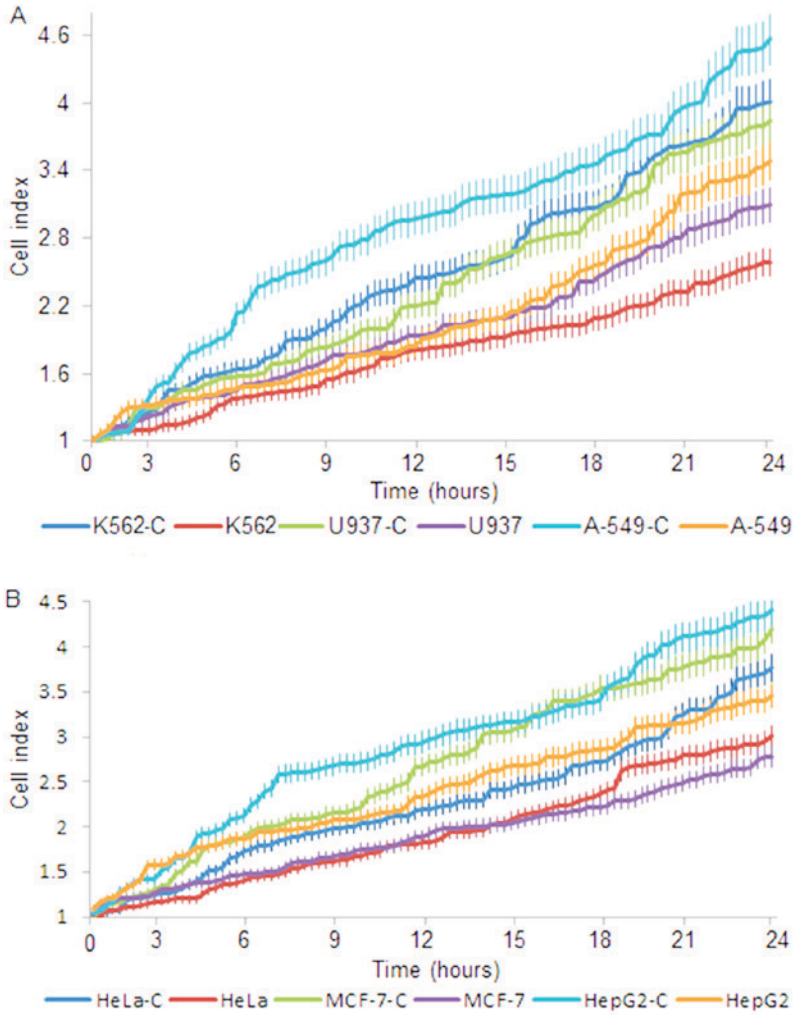

Figure 2. Real-time analysis of the effects of nobiliside D on the growth rate of human tumor cell lines. Six types of tumor cell lines were treated with nobiliside D. To observe cell lines clearly, the lines were assigned into two groups of equivalent size. (A) Results for human leukemic cell line K562, human leukemia cell line U937 and human lung cancer cell line A-549. (B) Results for human cervix carcinoma cell line HeLa, human breast cancer cell line MCF-7 and human liver carcinoma cell line HepG2. The cells were cultured for 24 h. The data are presented as mean values ± standard deviation. and n=5 in each group. K562-C, U937-C, A-549-C, HeLa-C, MCF-7-C and HepG2-C represent the control groups lacking nobiliside D treatment.

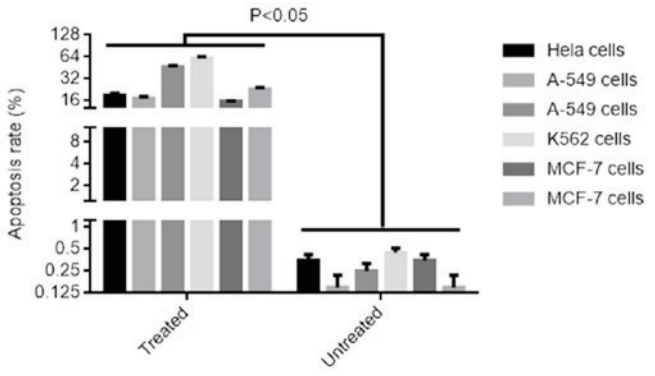

Figure 3. The effects of nobiliside D on the apoptosis rate of human cell lines. A greater apoptotic rate was observed in human tumor cell lines HeLa, A-549, K562, MCF-7, U937 and HepG2 treated with 0.5 μg/ml nobiliside D for 24 h, compared with untreated cells. Treated group, the cells were treated with nobiliside D. Untreated group, the cells were not treated by nobiliside D. P<0.05.

saponin family member nobiliside D. This extraction method achieves simultaneous extraction from *H. nobilis* Selenka, as well as improving the extraction efficiency and shortening the time of purification.

Saponins are established to have anti-tumor activity (13,14). Saponins are the main metabolites of sea cucumber (5,6) and currently >10 types of saponin have been isolated and purified (15,16). The abundance of novel compounds from sea cucumber suggests potential for biopharmaceutical applications (17-20). However, the saponin family member nobiliside D has been seldom isolated and its activity remains unknown. The present study indicates nobiliside D has clear inhibitory activities on the tumor cell lines tested and may offer potential for development as a novel drug for the treatment of various cancers.

However, studies are currently limited. For example, the detailed molecular mechanisms of the functioning of nobiliside D, which is a novel triterpenoid saponin, remain to be elucidated. In addition, there are numerous natural products from *H. nobilis* Selenka with anti-tumor activities and the activity of nobiliside D should be compared with these other compounds. Finally, the adverse effects of nobiliside D were not investigated, as these experiments would require *in vivo* models.

Collectively, the GC-MS and NMR analyses indicate that the novel compound nobiliside D was isolated from the sea cucumber H. *nobilis* Selenka in the present study. Nobiliside D demonstrated antiproliferative activities against the human tumor cell lines tested, particularly with human leukemic cell line K562 and breast cancer cell line MCF-7. The compound inhibits proliferation of these cells by promoting cellular apoptosis. The results suggest that nobiliside D could be developed as a potential drug for the treatment of breast cancer and human leukemia. However, the present study was limited to the cellular level and therefore, to verify the results, the compound requires testing in an animal model or preclinical trial. Further studies are required to enable full use of the therapeutic natural products of sea cucumber in the future.

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