MINISTRY OF EDUCATION AND TRAINING
VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

GRADUATE UNIVERSITY SCIENCE AND TECHNOLOGY

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STUDY ON CHEMICAL CONSTITUENTS, CYTOTOXIC ACTIVITIES OF THE SEA CUCUMBERS *Stichopus horrens* AND *Holothuria edulis* IN THE CENTRAL COAST OF VIETNAM SEA

Major: Organic chemistry
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SUMMARY OF CHEMISTRY DOCTORAL THESIS

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The thesis can be found in:

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INTRODUCTION

1. The urgency of the thesis

The Earth is sometimes called the “Ocean Planet”, with more than 70% of the surface of the Earth covered by salt water, and oceans are also home to over 90% of the Earth's habitable area and most of they are related to marine organisms. Therefore, it is not surprising to say that the marine environment is home to the largest species biodiversity with nearly 300.000 different species of plants, animals, and microorganisms... turning seas and oceans into extremely valuable natural resources, providing materials for many essential industries such as food, cosmetics, pharmaceutical chemicals...

Vietnam is blessed by nature with more than one million square kilometers of sea area, has a tropical monsoon climate, dense estuary density, is an ideal condition for marine organism diversity in species, and is rich in reserves. In the early 1970s, there was some research on natural compounds from marine organisms. However, compared to the potential of marine resources in our country, so far, domestic research works are too few and scattered, especially studies on the echinoderm.

To date, about 7.000 species of echinoderms have been recorded. Although the number of species is not much, the number of individuals of this phylum is very large, especially in clean and deepwater areas. The species of the echinoderm often have extremely interesting biological characteristics and an important component of the marine ecosystem. These marine organisms have always received interest of the researchers from various fields such as genetics biology, developmental studies, and especially research on valuable compounds with many medicinal values.

The chemotaxonomic relations observed in the phylum Echinodermata are particularly clear and included in five well-defined taxonomic classes Asteroidea (sea stars and starfish), Ophiuroidea (brittle stars and basket stars), Crinoidea (feather stars and sea lilies), Holothuroidea (sea cucumbers), and Echinoidea (sea urchins, sand dollars, and sea biscuits). Echinoderms are an important resource of natural products, which showed to have a significant positive impact on human health. Among them, one of the best examples is surely represented by sea cucumbers, a very promising group of marine invertebrates that during these last decades have gained the attention of researchers worldwide. Some of them are edible and have been used as food and folk medicine. Up to 2019, several sea cucumber species were reported on the chemical constituents and biological activities in the coastal zone of the Vietnam Sea. However, studies on the chemical constituents of Vietnamese Sea

cucumbers are limited. With the aim of investigating potential bioactive compounds from marine organisms in the echinoderm to create products that support medicinal benefits and public health promotion, the thesis namely "*Study on chemical constituents, cytotoxic activities of the sea cucumbers Stichopus horrens and Holothuria edulis in the central coast of Vietnam Sea*" was conducted with the following main contents:

2. The objectives of the thesis

♦ Isolation and determination of chemical structures of the compounds from the sea cucumbers *Stichopus horrens* and *Holothuria edulis.*

♦ Studied the cytotoxic activities of the isolated compounds to find the bioactive compounds.

3. The main contents of the thesis

♦ Isolation of compounds from the sea cucumbers *Stichopus horrens* and *Holothuria edulis* using various chromatographic separations.

♦ Determination of chemical structures of the isolated compounds.

♦ Evaluation of the cytotoxic activities of the isolated compounds from the sea cucumbers *S. horrens* and *H. edulis.*

4. Structure of the thesis

The Introduction/Preface (02 pages), Chapter 1: Overview (25 pages), Chapter 2: Research objective and Research methodology (07 pages), Chapter 3: Experiment and Empirical results (11 pages), Chapter 4: Discussions and Reusults (90 pages), Conclusions (02 pages). New findings of the thesis and publications within the scope of thesis (02 pages). Refences (139 references, 11 pages) and Content of spectrums (54 pages).

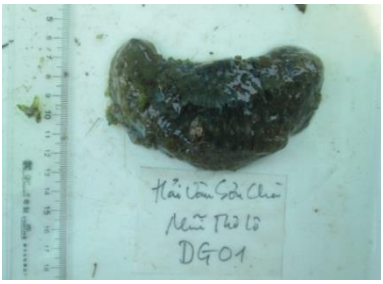
CHAPTER 1. OVERVIEW

This chapter presents the overview of domestic and international studies related to the chemical compositions and biological activities of the sea cucumber.

CHAPTER 2. RESEARCH OBJECTIVE AND RESEARCH METHODOLOGY

2.1. Research objective

The sample of two sea cucumbers *Stichopus horrens* and *Holothuria edulis* were collected at Hai Van - Son Tra, Thua Thien - Hue and Quang Nam (from May to August 2016). Two samples were identified by Prof. Do Cong Thung, the Institute of Marine Environment and Resources. The voucher specimens were deposited at the Institute of Marine Biochemistry and the Institute of Marine Environment and Resources, VAST, Vietnam.



Stichopus horrens Selenka, 1867

Holothuria edulis Lesson, 1830

2.2. Research methodology

2.2.1. Methods for extraction

The samples were cut into pieces and extracted three times with MeOH at room temperature (for three days) or in an ultrasonic bath (three times, each time 45 - 60 min). Evaporation of the solvent in vacuo obtained a residue, which was suspended in distilled water and partitioned in turn with *n*-hexane, CH_2Cl_2, and EtOAc.

2.2.2. Methods for metabolites isolation

Combining a number of chromatographic methods including thin-layer chromatography (TLC), column chromatography (CC), silica gel, RP-18, and Sephadex LH-20...

2.2.3. Methods for determination of the chemical structure of compounds

The general method used to determine the chemical structure of compounds is the combination between physical parameters and modern spectroscopic including melting point (Mp.), optical rotation ($[\alpha]_D$), electrospray ionization mass spectrometry (ESI-MS), and high-resolution ESI-MS (HR-ESI-MS), one/two-dimension nuclear magnetic resonance (NMR) spectra...

2.2.4. Methods for evaluation of cytotoxic activity

Cytotoxic activity of isolated compounds was evaluated against five human cancer cell lines, LNCaP (prostate cancer), MCF7 (breast cancer), KB (epidermoid carcinoma), HepG2 (hepatoma cancer), and SK-Mel-2 (melanoma) with minor modifications by Monks et al. Cell viability was evaluated using the SRB method according to the manufacturer's instructions. Experiments were performed in triplicate. The cell survival rates of the vehicle were calculated with GraphPad Prism. Each experiment was repeated at least three times independently, and IC_{50} values were presented as mean ± standard error of the mean. Elipticine was used as a positive control.

CHAPTER 3. EXPERIMENT AND EMPIRICAL RESULTS

3.1. Isolation of compounds from the sea cucumber *Stichopus horrens*

This part showed the extraction and isolation experiments of 17 compounds isolated from the sea cucumber *S. horrens*.

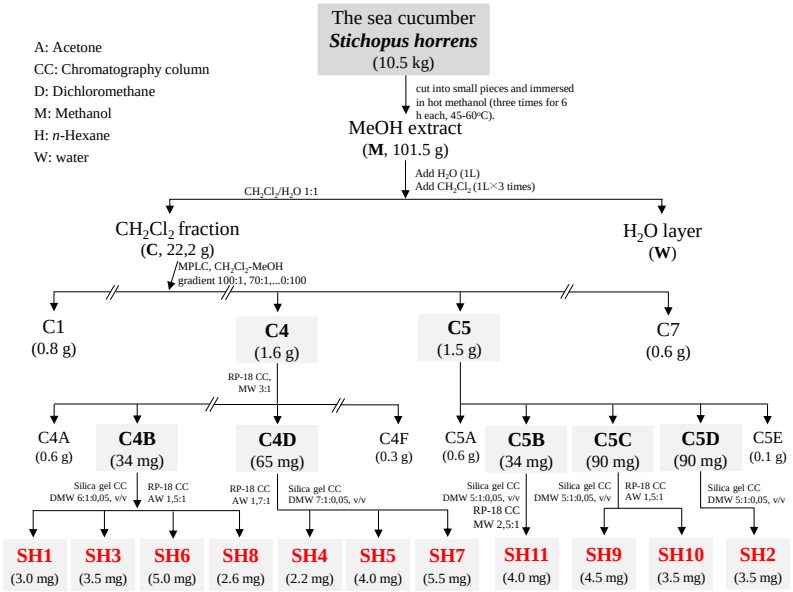


Figure 1. The partitioned MeOH extract and 11 compounds isolated from the sea cucumber *S. horrens*.

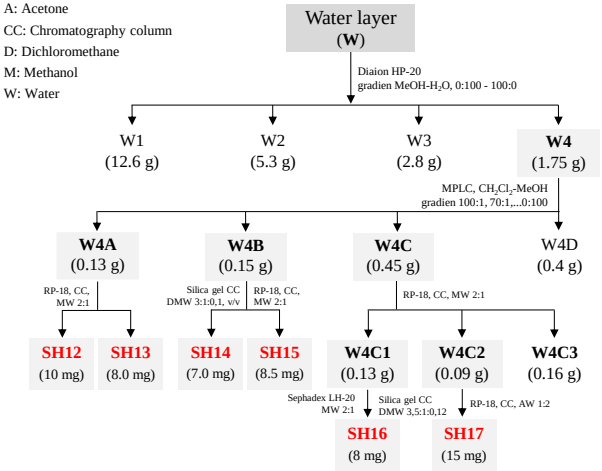


Figure 2. Six compounds isolated from the water layer of *S. horrens*.

3.2. Isolation of compounds from the sea cucumber *Holothuria edulis*

This section presents the process of isolating 8 compounds from the sea cucumber *H. edulis.*

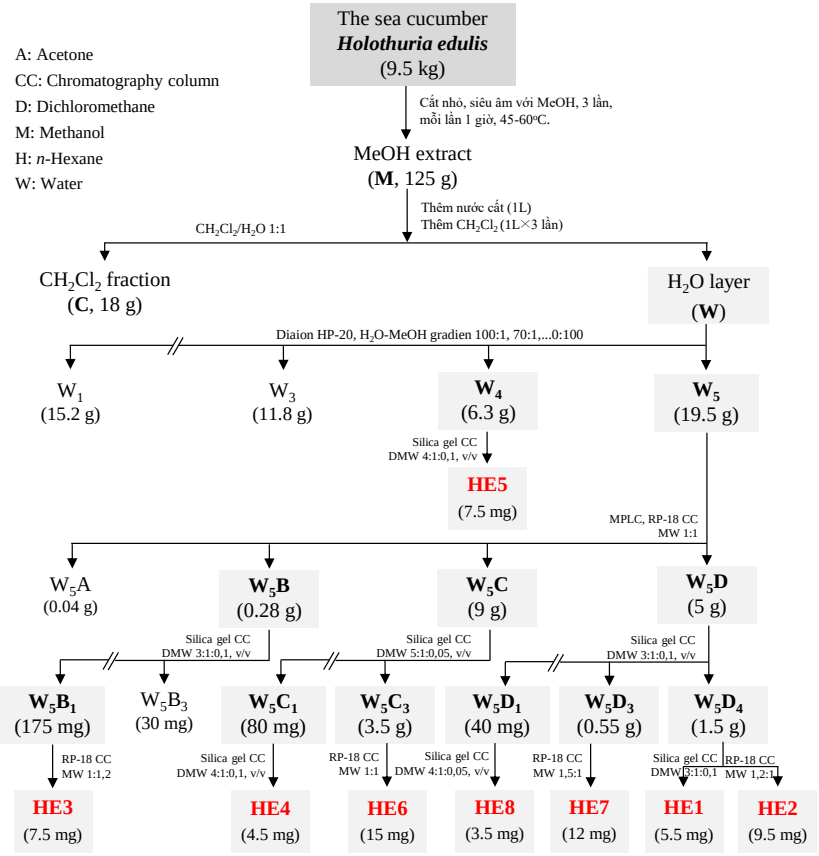


Figure 3. The partitioned MeOH extract and 8 compounds isolated from the sea cucumber *H. edulis*.

3.3. Physical properties and spectroscopic data of the isolated compounds

This section presents physical properties and spectroscopic data of 25 compounds from the sea cucumbers *S. horrens* and *H. edulis.*

CHAPTER 4. DISCUSSIONS

4.1. Determination of the chemical structure of compounds from the sea cucumber *Stichopus horrens*

This section presents the detailed results of spectral analysis and structure determination of 17 isolated compounds from the sea cucumber *Stichopus horrens*. Detailed methods for the determination of the chemical structure of a new compound are introduced in the following section.

4.1.1. Compound SH1: Stichorrenoside A (new compound)

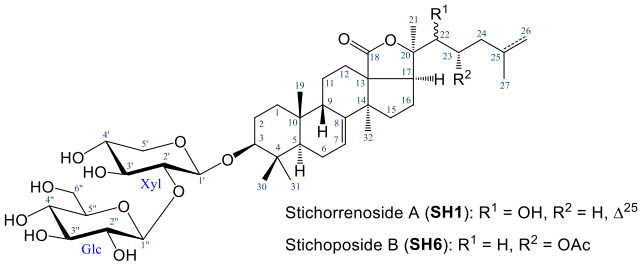


Figure 4. Structure of **SH1** and refence compound **SH6**.

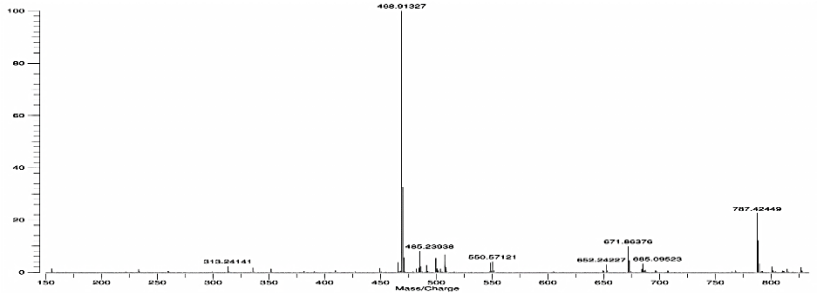


Figure 5. HR-ESI-MS spectrum of **SH1**.

Compound **SH1** was obtained as a white powder. Its HR-ESI-MS revealed a sodium adduct molecular ion peak at *m/z* 787.42449 $[M + Na]^+$, confirming a molecular formula of $C_{41}H_{64}O_{13}$. The NMR features indicated a triterpene diglycoside with typical signals of two anomeric carbons at δ_C 105.6 (C-1') and 105.4 (C-1''), which had HSQC correlations with the relevant anomeric protons at δ_H 4.85 (1H, overlapped signal, H-1') and 5.41 (1H, d, J = 7.5 Hz, H-1''). Detailed analysis of HSQC, HMBC, and ^{1}H-^{1}H COSY experiments led to assignment of the ^{1}H and ^{13}C NMR data for both sugar moieties (Table 7). These data were similar to those of stichoposide B, suggesting the disaccharide chain as *β*-D-glucopyranosyl-(1→2)-*β*-D-xylopyranoside. The attachment of the glucosyl moiety at C-2' of the xylosyl moiety was confirmed by an HMBC cross-peak of H-1'' (δ_H 5.41)

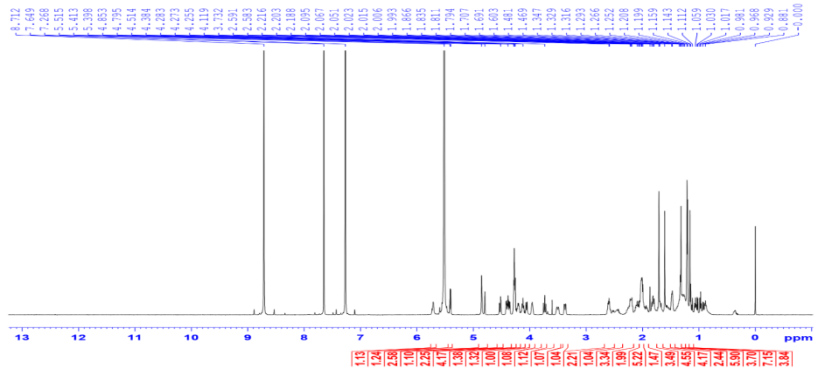


Figure 6. ^{1}H NMR spectrum of **SH1**.

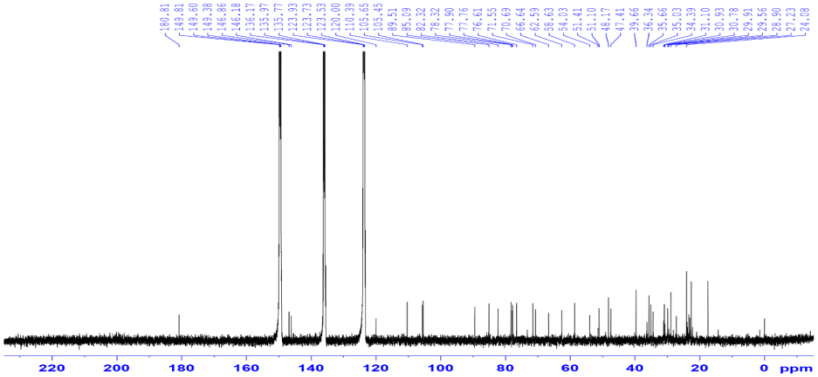


Figure 7. ^{13}C NMR spectrum of **SH1**.

with C-2' (δ_C 82.3). The D-configuration of both glucosyl and xylosyl moieties was assigned by analogy with stichoposide B, coexistence in *S. horrens*, and the reported holostane saponins. In addition, the ^{1}H and ^{13}C NMR data for the aglycone of **SH1** (Table 7) were also similar to those of stichoposide B, except for marked difference in signals for the side chain of these two compounds. The side chain of **SH1** contained signals of one oxymethine [δ_C 73.2 (C-22)/δ_H 4.06 (1H, br d, J = 9.5 Hz, H-22)], two methylenes [δ_C 31.1 (C-23) and 35.0 (C-24)/δ_H 1.92 (H-23a), 2.02 (H-23b), 2.21 (H-24a), and 2.59 (H-24b), each 1H, m], one terminal disubstituted double bond [δ_C 146.2 (C, C-25) and 110.4 (CH_2, C-26)/δ_H 4.79 (H-26a) and 4.85 (H-26b), each 1H, br s], and one *tert*-methyl [δ_C 22.6 (C-27)/δ_H 1.71 (3H, s, H-27)]. The HMBC cross-peaks of H-21 with C-17, C-20, and C-22; H-26 with C-24 and C-27; and those of H-27 with C-24, C-25, and C-26; as well as ^{1}H-^{1}H COSY correlations of H-22/H-23/H-24 (Fig. 10), clearly confirmed positions of

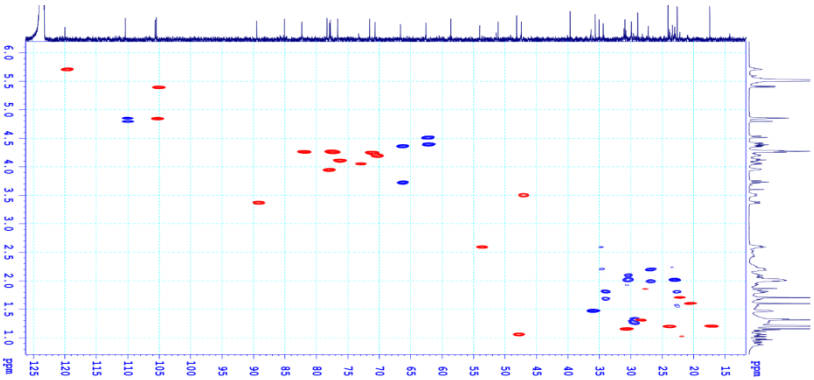


Figure 8. HSQC spectrum of **SH1**.

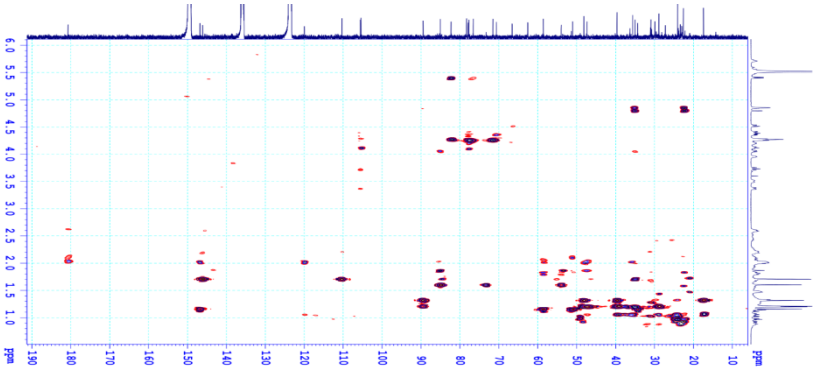


Figure 9. HMBC spectrum of **SH1**.

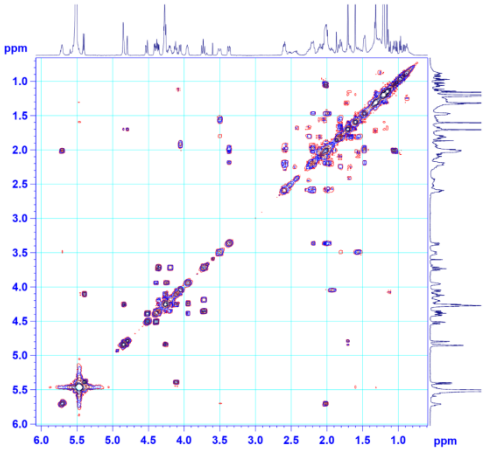


Figure 10. COSY spectrum of **SH1**.

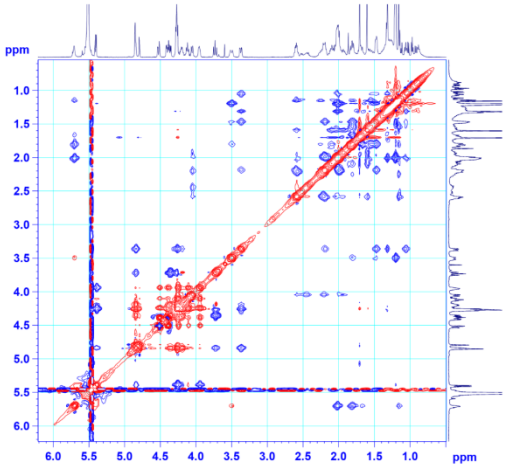


Figure 11. NOESY spectrum of **SH1**.

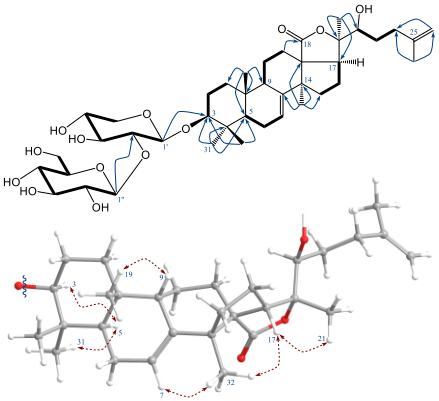


Figure 12. Keys COSY, HMBC, and NOESY correlations of **SH1**.

the oxymethine C-22, terminal double bond C-25/C-26, and *tert*-methyl C-27. The planar structure of **SH1** was clearly identified by detailed analysis of the other HMBC and ^{1}H-^{1}H COSY correlations (Figs. 9-10).

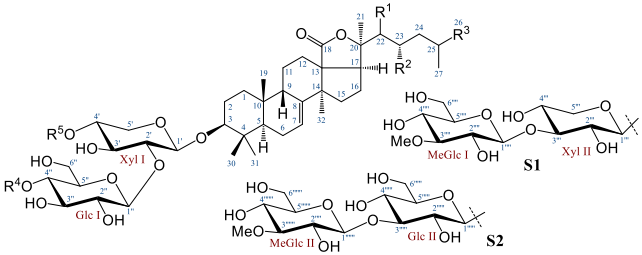
The configurations of the triterpene nucleus of **SH1** were assigned to be identical with those of stichoposide A (**SH5**), stichoposide B (**SH6**), 3*β*-*O*-[*β*-D-xylopyranosyl-(1→2)-*β*-D-xylopyranosyl]-23*S*-acetoxyholost-7-ene (**SH7**), and 3*β*-*O*-[*β*-D-xylopyranosyl-(1→2)-*β*-D-xylopyranosyl]-23*S*-hydroxyholost-7-ene (**SH8**), based on the agreement with their ^{1}H and ^{13}C NMR data, which were further confirmed by a ROESY experiment. The ROESY correlations of H-5 with H-3 and H_3-31 indicated the common *α*-orientation of H-3. Proton H-17 revealed ROESY correlations with both H-

Table 7. The NMR data of **SH1** and reference compound.

Pos.	[a]δ_C	δ_C[b,c]	δ_H[b,d] mult. (*J* in Hz)	HMBC
Aglycon				
1	36.4	36.3	1.47 m	
2	27.1	27.2	1.98 m/2.19 m	
3	89.3	89.5	3.37 dd (3.0, 11.5)	
4	39.6	39.6	-	
5	48.4	48.2	1.06 t (7.5)	
6	23.4	23.4	2.02 m	
7	120.0	120.0	5.71 br s	
8	146.7	146.8	-	
9	47.6	47.4	3.50 br d (13.5)	
10	35.7	35.7	-	
11	23.0	23.0	1.55 m/1.81 m	
12	30.6	30.7	2.02 m/2.10 m	
13	58.7	58.6	-	
14	51.4	51.1	-	
15	34.4	34.4	1.68 m/1.81 m	
16	25.0	23.9	2.23 m/2.45 m	
17	54.5	54.0	2.58 dd (4.5, 10.5)	
18	179.6	180.8	-	
19	24.0	24.0	1.20 s	1, 5, 9, 10
20	82.9	85.1	-	
21	27.1	21.0	1.60 s	17, 20, 22
22	44.3	73.2	4.06 br d (9.5)	
23	68.7	31.1	1.92 m/2.02 m	
24	45.5	35.0	2.21 m/2.59 m	
25	24.8	146.1	-	
26	23.1	110.4	4.85 br s/4.79 br s	
27	22.3	22.6	1.71 s	24, 25, 26
30	17.4	17.4	1.21 s	3, 4, 5, 31
31	29.0	28.9	1.33 s	3, 4, 5, 30
32	30.9	30.9	1.16 s	8, 13, 14, 15
Xyl				
1'	105.5	105.6	4.85*	3
2'	82.9	82.3	4.26*	
3'	77.8	77.9	4.26*	
4'	70.8	70.7	4.20 m	
5'	66.4	66.6	3.73 dd (11.0, 10.5)	
			4.37 dd (5.0, 11.0)	
Glc				
1"	105.9	105.5	5.41 d (7.5)	2'
2"	76.5	76.6	4.12 dd (7.5, 9.0)	
3"	77.9	77.8	4.26*	
4"	72.0	71.6	4.25*	
5"	77.8	78.3	3.95 m	
6"	63.0	62.6	4.40 dd (5.0, 12.0)	
			4.53 dd (2.5, 12.0)	

*[a]δ_C of stichoposide B, [b]pyridine-d_5, [c]125 MHz, and [e]500 MHz. *Overlapped signals.*

21 and H_3-32, confirming α-orientation of H-17. Moreover, the β-orientation of H-9 was assigned by a spatial proximity of H-9 with H-19. From all above evidence, the structure of **SH1** was elucidated as 3β-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl]-22ξ-hydroxy holost-7,25-diene.



Stichorrenoside A (**SH1**): R^1 = OH, R^2 = H_2, R^3 = CH_2, R^4 = R^5 = H

Stichorrenoside B (**SH2**): R^1 = OH, R^2 = H_2, R^3 = CH_2, R^4 = $NaSO_3$, R^5 = H

Stichorrenoside C (**SH3**): R^1 = H, R^2 = OAc, R^3 = CH_2, R^4 = R^5 = H

Stichorrenoside E (**SH9**): R^1 = H, R^2 = OAc, R^3 = CH_2, R^4 = S1, R^5 = H

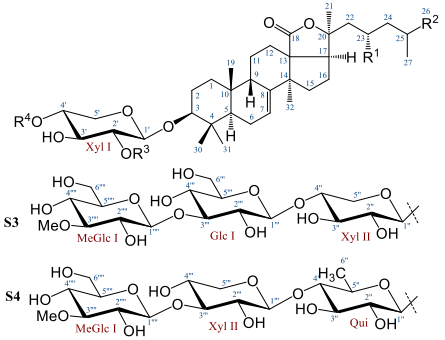
Thelenotoside B (**SH10**): R^1 = H, R^2 = OAc, R^3 = CH_3, R^4 = S1, R^5 = H

Deacetyl thelenotoside B (**SH11**): R^1 = H, R^2 = OH, R^3 = CH_3, R^4 = S1, R^5 = H

Stichloroside B_1 (**SH12**): R^1 = H, R^2 = OAc, R^3 = CH_3, R^4 = S1, R^5 = S2

Stichloroside B_2 (**SH13**): R^1 = H, R^2 = OAc, R^3 = CH_2, R^4 = S1, R^5 = S2

Deacetylstichloroside B_1 (**SH14**): R^1 = H, R^2 = OH, R^3 = CH_2, R^4 = S1, R^5 = S2



Stichorrenoside D (**SH4**): R^1 = OAc, R^2 = CH_2, R^3 = Xyl, R^4 = H

Stichoposide A (**SH5**): R^1 = OAc, R^2 = CH_3, R^3 = Qui, R^4 = H

Stichoposide B (**SH6**): R^1 = OAc, R^2 = CH_3, R^3 = Glc, R^4 = H

3β-*O*-[β-D-xylopyranosyl-(1-2)-β-D-xylopyranosyl]-23*S*-acetoxyholost-7-ene (**SH7**): R^1 = OAc, R^2 = CH_3, R^3 = Xyl, R^4 = H

3β-*O*-[β-D-xylopyranosyl-(1-2)-β-D-xylopyranosyl]-23*S*-hydroxyholost-7-ene (**SH8**): R^1 = OH, R^2 = CH_3, R^2 = Xyl, R^4 = H

Stichloroside A_1 (**SH15**): R^1 = OAc, R^2 = CH_3, R^3 = S3, R^4 = S2

Deacetylstichloroside A_1 (**SH16**): R^1 = OH, R^2 = CH_3, R^3 = S3, R^4 = S2

Deacetyltichloroside C_1 (**SH17**): R^1 = OH, R^2 = CH_3, R^3 = S4, R^4 = S2

Figure 67. The structures of compounds isolated from *S. horrens*.

4.1. Determination of the chemical structure of compounds from the sea cucumber *Stichopus horrens*

This section presents the detailed results of spectral analysis and structure determination of 08 isolated compounds from the sea cucumber *Holothuria edulis*. Detailed methods for the determination of the chemical structure of a new compound are introduced in the following section.

4.1.2. Compound HE1: Holothurin A_5 (new compound)

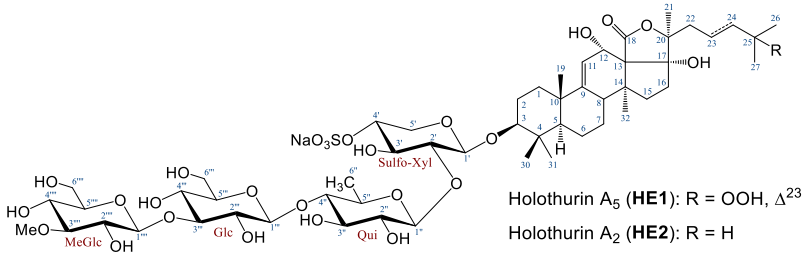


Figure 68. Structure of **HE1** and reference compound **HE2**.

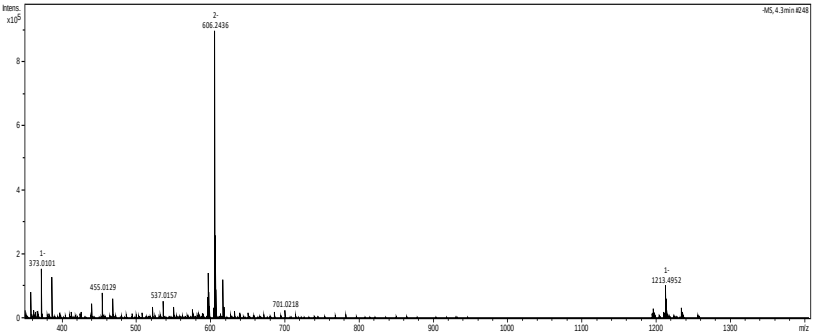


Figure 69. HR-ESI-MS spectrum of **HE1**.

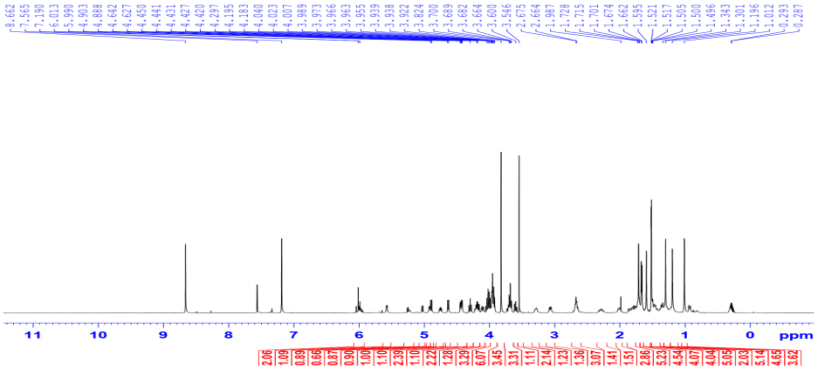


Figure 70. ^{1}H NMR spectrum of **HE1**.

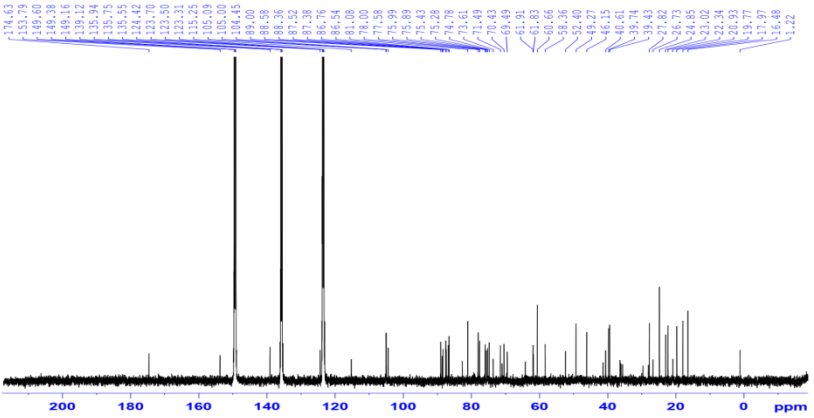


Figure 71. ^{13}C NMR spectrum of **HE1**.

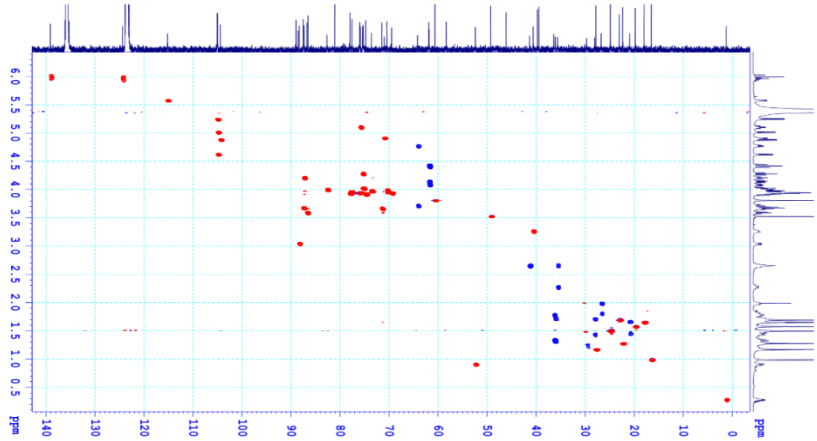


Figure 72. HSQC spectrum of **HE1**.

Compound **HE1** was isolated as a white amorphous powder. Its molecular formula was determined as $C_{54}H_{85}NaO_{28}S$ from the $[M - Na]^-$ ion peak at *m/z* 1213.49521 in the negative HR-ESI-MS as well as the $[M + Na]^+$ sodium adduct ion peak at *m/z* 1259 in the positive ESI-MS. The fragment ion peak at *m/z* 97 $[HSO_4]^-$ in the negative ESI-MS/MS spectrum of the ion at *m/z* 1213 $[M - Na]^-$ showed the presence of a sulfate group in **HE1**. The NMR features indicated a holostane-type saponin, one of the main constituents of *Holothuria* sea cucumbers. The ^{1}H NMR spectrum exhibited typical signals of four anomeric protons (each 1H, d, J = 7.5 or 8.0 Hz) at δ_H 4.62 (H-1'), 5.01 (H-1"), 4.88 (H-1"') and 5.25 (H-1""), which had HSQC correlations with the corresponding anomeric carbons at δ_C 105.0 (C-1' and C-1"), 104.4 (C-1"') and 105.1 (C-1""), confirming presence

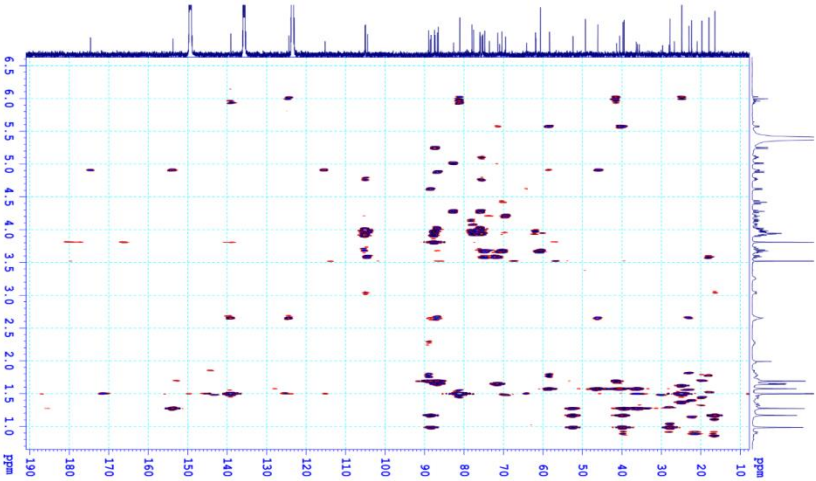


Figure 73. HMBC spectrum of **HE1**.

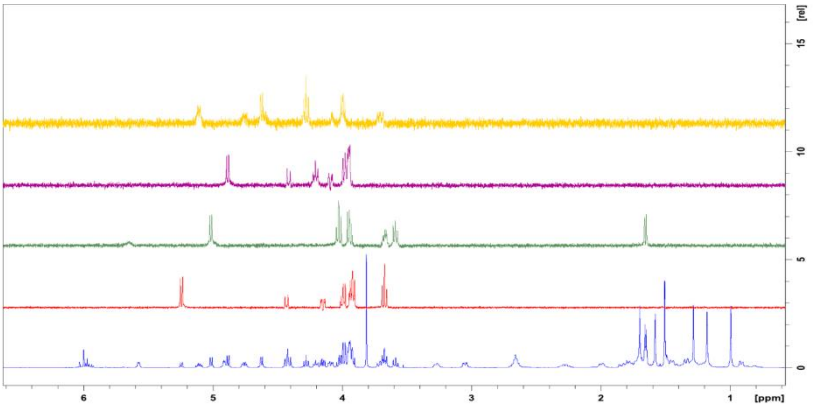


Figure 74. 1D TOCSY spectrum of **HE1**.

of four sugar moieties in **HE1**. The completed ^{1}H and ^{13}C NMR data for all four sugar moieties (Table 24) were assigned by detailed analysis of the HSQC, HMBC, ^{1}H-^{1}H COSY, 1D and 2D TOCSY experiments. These data were similar to those of **HE2** and **HE3**, suggesting the tetrasaccharide chain as 3-*O*-methyl-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→4)-*β*-D-quinovo pyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranoside, one common tetraglycoside chain of saponins obtained from *Holothuria* species. The HMBC cross-peaks of H-1"" with C-3"', H-1"' with C-4", and H-1" with C-2' confirmed the sequence of sugar moieties in the tetrasaccharide chain of **HE1**. The sequence of the carbohydrate chain was further supported by negative ESI-MS/MS which contained the fragment ion peaks at *m/z*

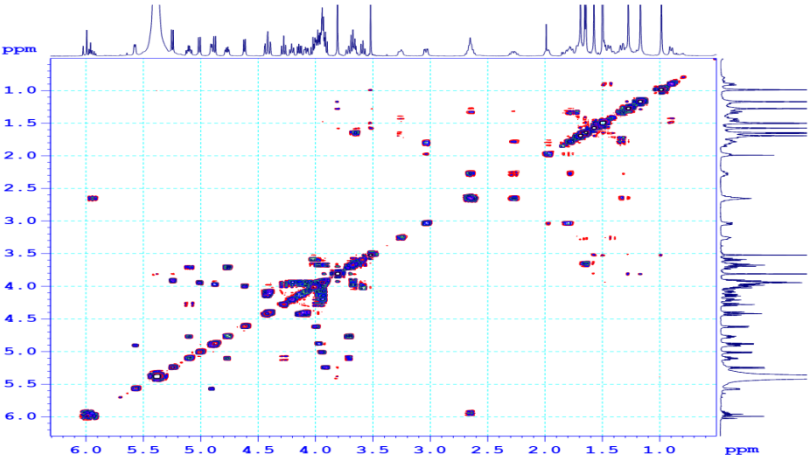


Figure 75. COSY spectrum of **HE1**.

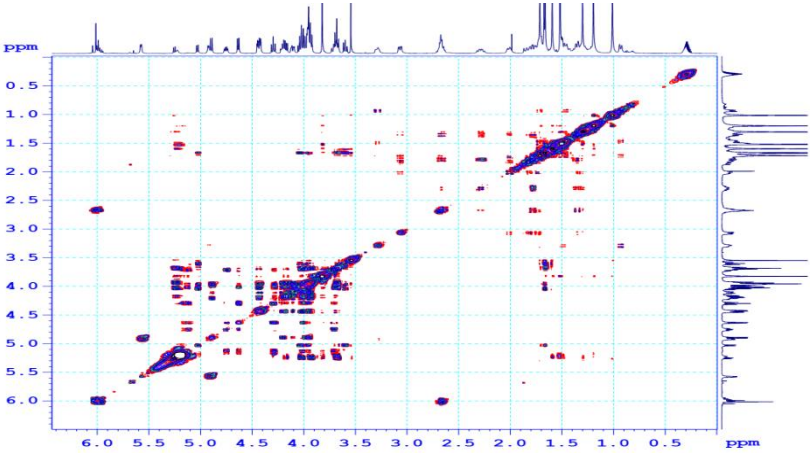


Figure 76. 2D TOCSY spectrum of **HE1**.

1005 [1181 - $C_7H_{12}O_5$]$^-$, 843 [1181 - $C_7H_{12}O_5$ - $C_6H_{10}O_5$]$^-$, 825 [1181 - $C_7H_{12}O_5$ -$C_6H_{10}O_5$ - H_2O]$^-$, 697 [1181 -$C_7H_{12}O_5$ - $C_6H_{10}O_5$ - $C_6H_{10}O_4$]$^-$, 695 [carbohydrate chain - Na]$^-$, 519 [(carbohydrate chain - Na) - $C_7H_{12}O_5$]$^-$, 357 [(carbohydrate chain - Na) - $C_7H_{12}O_5$ - $C_6H_{10}O_5$]$^-$, 211 [(carbohydrate chain - Na) - $C_7H_{12}O_5$ - $C_6H_{10}O_5$ - $C_6H_{10}O_4$]$^-$ corresponding to the successive losses of *O*-methyl-hexose, hexose, deoxyhexose units, carbohydrate chain and its fragmentation. The D-configuration of all the four sugar moieties was assigned on the basis of biosynthetic reasons, analogy with **HE2**, **HE3**, and **HE5**, coexistence in the sea cucumber *H. edulis*, and reported holostane saponins from the sea cucumbers. In addition, the ^{1}H and ^{13}C NMR data for

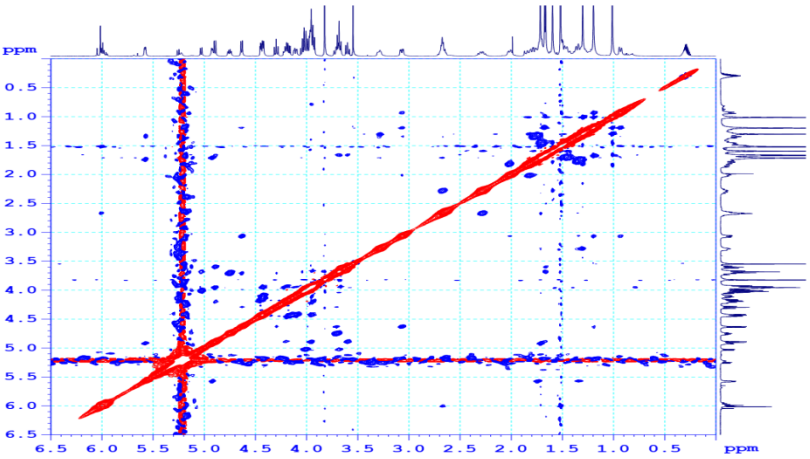


Figure 77. NOESY spectrum of **HE1**.

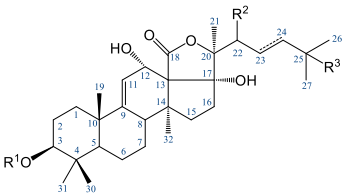
the aglycone of **HE1** were similar to those of holothurin A_2 (**HE2**) (Table 24), except for signals of the side chains with presence of a methylene [δ_C 41.3 (C-22)/δ_H 2.64 (2H, m, H-22)], a *trans* disubstituted double bond [δ_C 124.4 (C-23)/δ_H 5.95 (1H, ddd, J = 6.5, 9.0, 15.5 Hz, H-23) and δ_C 139.1 (C-24)/δ_H 6.01 (1H, d, J = 15.5 Hz, H-24)], an oxygenated quaternary carbon [δ_C 81.1 (C-25)], and two *tert*-methyl groups [δ_C 24.8 (C-26 and C-27)/δ_H 1.49 (H_3-26) and 1.50 (H_3-27), each 3H, s] in **HE1**. The ^{13}C NMR peak at C-25 of **HE1** was strongly shifted downfield at δ_C 81.1 relative to that of leucospilotaside A (**HE5**) at δ_C 69.0 and holothurin B_4 at δ_C 69.5, indicating presence of a hydroperoxy group at this arbon. The COSY correlations of H-22/H-23/H-24 as well as HMBC cross-peaks of H_3-21 with C-17, C-20 and C-22; H_3-26 and H_3-27 with C-24 and C-25 clearly elucidated the side chain of **HE1** as shown in the Figure 148. The relative configurations of the triterpene skeleton of **HE1** were assigned to be identical to those of **HE2**–**HE8** from biosynthesis view with coexistence of them in the sea cucumber *H. edulis*. This assignment was also supported by the agreement of their ^{1}H and ^{13}C NMR data as well as by ROESY experiment (Fig. 152). Finally, attachment of the tetrasaccharide chain at C-3 of the aglycon was demonstrated by HMBC cross-peak of the anomeric proton H-1' with C-3. Thus, the structure of **HE1** was established to be 3*β*-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→4)-*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-25-hydro peroxyholost-9(11),23*E*-diene-12*α*,17*α*-diol. The presence of hydroperoxy groups in triterpene saponins is relatively rare. To the best of our knowledge, this is the first report of this group in triterpene saponins obtained from sea cucumbers to date.

Table 24. The NMR data of **HE1** and reference compound.

Pos.	[a]δ_C	δ_C[b,c]	δ_H[b,d] mult. (*J* in Hz)	HMBC
Aglycon				
1	36.5	36.1	1.30 m/1.71 m	
2	27.2	26.7	1.80 m/1.99 m	
3	88.8	88.4	3.04 dd (4.0, 11.5)	
4	40.1	39.7	-	
5	52.8	52.4	0.90 br d (11.0)	
6	21.3	20.9	1.45 m/1.66 m	
7	28.5	28.1	1.43 m/1.70 m	
8	41.0	40.6	3.27 dd (5.5, 12.0)	
9	154.4	153.8	-	
10	39.9	39.4	-	
11	115.4	115.2	5.57 br d (5.0)	
12	71.0	71.0	4.91 d (5.0)	18
13	58.7	58.4	-	
14	46.4	46.1	-	
15	36.9	36.4	1.33 m/1.78 m	
16	39.2	35.6	2.28 m/2.64 m	
17	87.5	89.0	-	
18	173.7	174.6	-	
19	22.7	22.3	1.28 s	1, 5, 9, 10
20	92.4	86.5	-	
21	21.5	23.0	1.69 s	17, 20, 22
22	208.0	41.3	2.64 m	
23	34.1	124.4	5.95 ddd (6.5, 9.0, 15.5)	
24	34.7	139.1	6.01 d (15.5)	
25	81.4	81.1	-	
26	26.2	24.9	1.49 s	24, 25, 27
27	26.1	24.8	1.50 s	24, 25, 26
30	16.9	16.5	0.98 s	3, 4, 5, 31
31	28.2	27.8	1.19 s	3, 4, 5, 30
32	20.0	19.8	1.57 s	8, 13, 14, 15
Sulfo-Xyl				
1'	105.4	105.0	4.62 d (7.5)	3
2'	83.5	82.6	4.00 dd (7.5, 9.0)	
3'	75.4	75.4	4.28 t (9.0)	
4'	76.5	75.9	5.10 m	
5'	64.4	64.2	3.71 dd (11.0, 11.5)	
			4.77 dd (5.0, 11.5)	
Qui				
1"	105.6	105.0	5.01 d (8.0)	2'
2"	75.6	75.9	3.94 dd (8.0, 9.0)	
3"	75.9	75.2	4.02 t (9.0)	
4"	86.8	86.7	3.59 t (9.0)	
5"	72.1	71.4	3.67 dd (9.0, 6.0)	

Pos.	[a]δ_C	δ_C[b,c]	δ_H[b,d] mult. (*J* in Hz)	HMBC
6"	18.2	17.9	1.64 d (6.0)	4", 5"
Glc				
1"'	105.0	104.4	4.88 d (8.0)	4"
2"'	74.2	73.6	3.97 dd (8.0, 9.0)	
3"'	88.1	87.3	4.21 t (9.0)	
4"'	69.6	69.5	3.93*	
5"'	77.8	77.6	3.94*	
6"'	61.9	61.8	4.08 dd (5.5, 12.0)	
			4.41 br d (12.0)	
OMe-Glc				
1"''	106.0	105.1	5.25 d (8.0)	3"'
2"''	75.1	74.8	3.92 dd (8.0, 9.0)	
3"''	88.1	87.5	3.67 t (9.0)	
4"''	70.7	70.4	3.98 t (9.0)	
5"''	78.4	78.0	3.94 m	
6"''	62.2	61.9	4.14 dd (5.5, 12.0)	
			4.43 dd (2.0, 12.0)	
OMe	61.0	60.7	3.81 s	3"''

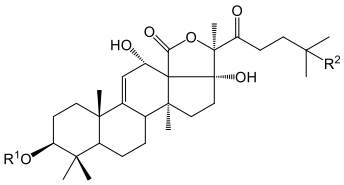
[a]δ_C *of marmoroside C,* [b]*pyridine-*d_5, [c]*125 MHz, and* [d]*500 MHz. *Overlapped signals.*



Holothurin A_5 (**HE1***): Δ^{23}, R^1 = S1, R^2 = H_2, R^3 = OOH

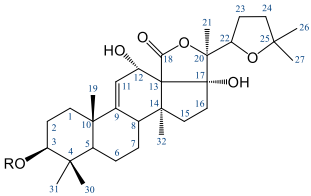
Holothurin A_2 (**HE2**): R^1 = S1, R^2 = H_2, R^3 = H

Marmoroside C (**HE3**): R^1 = S1, R^2 = O, R^3 = OAc



Moebioside A (**HE4**): R^1 = S2, R^2 = OAc

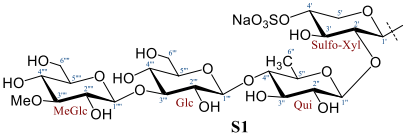
Leucospilotaside A (**HE5**): R^1 = S2, R^2 = OH



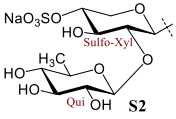
Holothurin A (**HE6**): R = S1

Holothurin B (**HE7**): R = S2

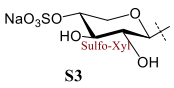
Leucospilotaside C (**HE8**): R = S3



S1



S2



S3

Figure 86. The structures of compounds isolated from *H. edulis*.

4.3. Biological activities of isolated compounds

4.3.1. Cytotoxic activity of compounds isolated from the sea cucumbers

Cytotoxicity testing method is carried out to evaluate the ratio of living cells and dead cells after treatment cells with tested samples. This is a basic method to screening new compounds for the development of anti-cancer agents. The SRB method was used to evaluate the cytotoxic activity of all the isolated compounds against five human cancer cell lines HepG2, KB, LNCaP, MCF7, and SK-Mel-2.

As the obtained results (Table 32), stichorrenoside D (**SH4**), stichoposide A (**SH5**), and 3*β*-*O*-[*β*-D-xylopyranosyl-(1→2)-*β*-D-xylopyranosyl]-23*S*-acetoxyholost-7-ene (**SH7**) showed strong cytotoxicity against all five tested cancer cell lines with IC_{50} values ranging from 1.92 ± 0.61 to 3.13 ± 0.40 μM, comparable to that of the positive control (ellipticine, IC_{50} values ranging from 1.34 ± 0.16 to 1.95 ± 0.20 μM). Significant effect against all five tested cancer cell lines was observed for stichorrenoside C (**SH3**) and stichoposide B (**SH6**) with IC_{50} values ranging from 5.28 ± 0.25 to 11.00 ± 0.20 μM, whereas moderate activity was observed for the other compounds with IC_{50} values ranging from 33.48 ± 2.20 to 59.31 ± 4.77 μM. Generally, the number, length, and the type and linkage variation in sugar moieties as well as the side chain structures significantly influence the bioactivity of saponins. Besides, compounds **HE2**, **HE6**, and **HE7** showed strong cytotoxicity (IC_{50} values ranging from 0.75 ± 0.09 to 3.66 ± 0.41 μM) against all five cell lines, comparable to that of the positive control. Compounds **HE1**, **HE3**, and **HE4** showed moderate cytotoxic effect against these cell lines with IC_{50} values ranging from 46.65 ± 2.28 to 82.75 ± 3.91 μM, whereas **HE5** and **HE8** revealed weak (IC_{50} ~ 90 μM) or less effect (IC_{50} > 100 μM).

Consideration of the chemical structures of compounds suggested that the presence of quinovose and second xylose moieties in the disaccharide chains and the acetoxy group in the side chains might play an important role for the cytotoxic activity of these compounds. Moreover, the presence of acetoxy groups was previously reported to enhance cytotoxic potency of some saponins. However, this is dependent on concentration and other specific biological activities have been noted. There is a close relationship between the chemical structure of saponins and their biological activities. Observations from numerous studies confirm that the biological activity of saponins is influenced both by the aglycone and the carbohydrate moiety. It has been stated that having a linear carbohydrate chain is essential for the biological activity of saponins resulting in modifying the cellular membrane.

Table 32. Inhibitory effects of isolated compounds on the growth of five human cancer cell lines.

Comp.	IC_{50} values (μM)				
	LNCaP	MCF7	KB	HepG2	SK-Mel-2
SH1	51.56 ± 4.19	41.86 ± 4.21	33.48 ± 2.20	37.93 ± 4.55	43.53 ± 1.66
SH2	51.47 ± 1.45	59.52 ± 4.58	45.44 ± 2.00	55.28 ± 4.75	67.15 ± 4.70
SH3	10.06 ± 0.47	7.25 ± 0.78	11.00 ± 0.20	7.03 ± 0.88	10.82 ± 0.72
SH4	3.13 ± 0.40	2.11 ± 0.27	2.36 ± 0.36	1.92 ± 0.61	2.27 ± 0.22
SH5	3.02 ± 0.33	2.12 ± 0.30	2.82 ± 0.29	2.97 ± 0.37	2.70 ± 0.23
SH6	7.60 ± 0.30	6.36 ± 0.22	8.86 ± 0.24	5.28 ± 0.25	5.77 ± 0.48
SH7	2.70 ± 0.28	2.08 ± 0.44	3.11 ± 0.32	2.04 ± 0.73	2.21 ± 0.19
SH8	59.31 ± 4.77	52.24 ± 2.96	48.42 ± 5.22	53.75 ± 5.08	41.94 ± 1.74
SH9	9.35 ± 0.23	8.95 ± 0.49	7.48 ± 0.22	6.87 ± 0.25	10.59 ± 0.44
SH10	1.90 ± 0.13	1.56 ± 0.23	0.95 ± 0.08	1.33 ± 0.10	1.14 ± 0.11
SH11	11.62 ± 1.05	11.45 ± 0.30	10.72 ± 0.18	8.45 ± 0.23	10.25 ± 0.61
SH12	**0.18 ± 0.02**	**0.13 ± 0.01**	**0.14 ± 0.02**	**0.10 ± 0.01**	**0.14 ± 0.02**
SH13	1.40 ± 0.21	0.85 ± 0.23	1.36 ± 0.18	0.96 ± 0.16	1.31 ± 0.19
SH14	1.45 ± 0.16	1.08 ± 0.04	1.51 ± 0.22	1.16 ± 0.06	1.38 ± 0.08
SH15	1.24 ± 0.08	1.04 ± 0.14	1.22 ± 0.14	1.20 ± 0.13	0.85 ± 0.10
SH16	1.32 ± 0.18	1.12 ± 0.08	1.63 ± 0.20	1.18 ± 0.11	1.25 ± 0.05
SH17	**0.27 ± 0.01**	**0.24 ± 0.04**	**0.29 ± 0.04**	**0.34 ± 0.04**	**0.26 ± 0.06**
HE1	66.22 ± 6.32	49.08 ± 6.44	46.65 ± 2.28	57.53 ± 6.27	63.53 ± 3.49
HE2	0.96 ± 0.09	0.81 ± 0.07	0.75 ± 0.09	0.76 ± 0.06	0.84 ± 0.05
HE3	82.75 ± 3.91	76.45 ± 6.29	67.31 ± 6.93	75.76 ± 7.60	68.55 ± 3.18
HE4	57.61 ± 5.54	55.99 ± 6.43	64.72 ± 4.94	59.59 ± 3.38	61.65 ± 5.67
HE5	>100	91.47 ± 3.30	91.27 ± 5.41	93.56 ± 4.95	>100
HE6	1.30 ± 0.18	2.29 ± 0.47	1.79 ± 0.33	2.03 ± 0.49	2.49 ± 0.21
HE7	2.74 ± 0.29	3.35 ± 0.47	2.75 ± 0.31	2.63 ± 0.28	3.66 ± 0.41
HE8	>100	>100	>100	>100	>100
Elipticine	1.95 ± 0.20	1.34 ± 0.16	1.79 ± 0.28	1.38 ± 0.28	1.91 ± 0.20

Elipticine was used as a positive control. Results are the means ± SD of independent experiments in triplicate.

4.3.2. The ability to kill tumor cells of deacetylstichloroside C_1 (SH17) by mechanism of action on biological targets

*a. Effects of compound **SH17** on MCF7 cell cycle*

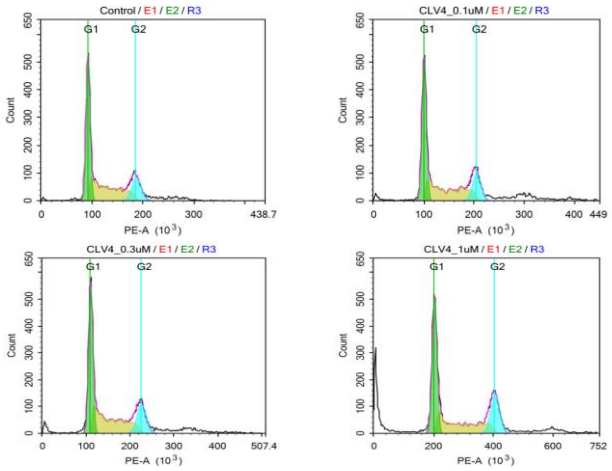


Figure 87. Effects of compound **SH17** on MCF7 cell cycle.

Table 33. The percentage (%) of MCF7 cells in phase G_0/G_1, S, G_2/M, and apoptosis (sub-G_1) after 48h of induction with **SH17** compound at concentrations of 0.1, 0.3, and 1.0 μM.

Compounds	**The percentage of MCF7 cells in phase (%)**			
	G_0/G_1	**S**	**G_2/M**	**sub-G_1**
Control	40.33	34.96	15.86	1.23
SH17_0.1 μM	37.29	31.18	14.82	2.86
SH17_0.3 μM	35.84	30.64	16.09	3.79
SH17_1.0 μM	32.52	24.82	18.05	16.88

When the cell cycle distribution was analyzed after 48 h of treatment with compound **SH17**, an increase in sub-G_1 hypodiploid cells (2.86, 3.79, and 16.88%) was observed at concentrations of 0.1, 0.3, and 1.0 μM (Figure 87 and Table 33). Since nuclear morphological changes are critical markers of cell apoptosis, we performed Hoechst staining to confirm the induction of nuclear morphological changes in the tested samples. Simultaneously, the percentage of cells in the G_0/G_1 and S phases gradually decreased with the increase in the concentration of compound **SH17**. It shows that compound **SH17** arrests cells in the sub-G_1 stage according to the concentration. This shows to suggest compound **SH17** effects the cell cycle in the sub-G_1 phase.

*b. Effect of **SH17** on the apoptosis of MCF7 cells*

The results showed that after treatment cells with compound **SH17** at a concentration of 1.0 μM moved to the apoptosis stage with a rate of 36.08%. Meanwhile, the percentage of cells that spontaneously died in the

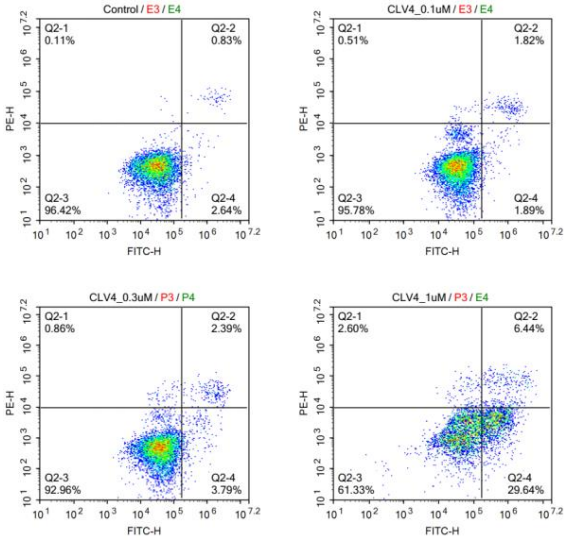


Figure 88. Effect of **SH17** on the apoptosis of MCF7 cells.

control sample was only 3.47% (Figure 88). This suggested that compound **SH17** induced MCF7 cell death.

*c. Effect of **SH17** compound on MCF7 cell morphological changes*

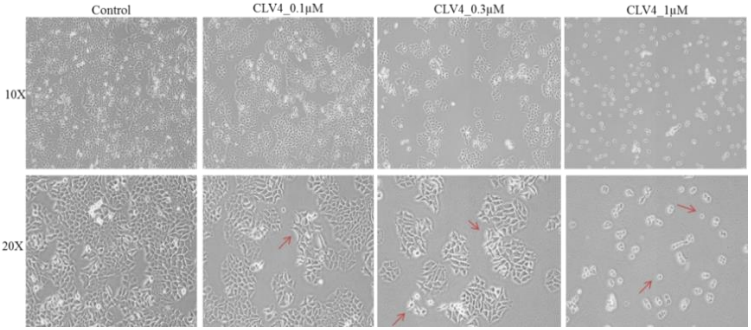


Figure 89. Effect of **SH17** on MCF7 cell morphological changes.

The fact that the compound induces apoptosis on MCF7 cells was confirmed by the change of MCF7 cell morphology after treatment with compound **SH17** at concentrations of 0.1, 0.3, and 1.0 μM. It can be clearly seen that the cell density decreases with increasing concentration of this compound. In particular, at a concentration of 1.0 μM, the maximally concentrated chromatids became adherent to the membrane surrounding the cell nucleus (Figure 89).

CONCLUSIONS

- ***Chemical composition investigations***

The isolation and structural elucidation of interesting 25 triterpene glycosides are facilitated by the continual development of techniques such as using various chromatographic separations, spectroscopic analyses, for instance, nuclear magnetic resonance (NMR) and mass spectrum (MS).

1. Five new compounds, stichorrenosides A-E (**SH1**-**SH4** and **SH9**), were isolated from the sea cucumber *Stichopus horrens*.

2. A new holothurin A_5 (**HE1**) was reported from the sea cucumber *H. edulis*.

- ***Biological activity investigations***

1. All the isolated compounds were evaluated for their cytotoxic activities against five human cancer cell lines LNCaP (prostate cancer), MCF7 (breast cancer), KB (epidermoid carcinoma), HepG2 (hepatoma cancer), and SK-Mel-2 (melanoma) by SRB method. As the results, 23/25 compounds show cytotoxicity against all five tested cancer cell lines. Only compound **HE5** showed weak cytotoxicity against all three tested cancer cell lines (MCF7, KB, and HepG2), whereas **HE8** revealed less effect (IC_{50} > 100 μM) against five cell lines. Compounds stichloroside B_1 (**SH12**) and deacetylstichloroside C_1 (**SH17**) showed strong cytotoxicity against all five cell lines (with IC_{50} values ranging from 0.10 to 0.34 μM), comparable to that of the positive control (ellipticine, IC_{50} values ranging from 1.34 to 1.95 μM),

2. The ability of deacetylstichloroside C_1 to kill cancer cells has been evaluated according to the mechanism of action on biological targets. The results showed that this compound affects the cell cycle progression in the sub-G_1 phase and induces MCF7 cell apoptosis.

RECOMMENDATIONS

1. Stichloroside B_1 (**SH12**) and deacetylstichloroside C_1 (**SH17**) exhibited strong cytotoxicity against all five tested cancer cell lines. Preliminary findings revealed that saponins provide their anticancer activities through a number of mechanisms including arresting cell cycles, induction of apoptosis, blocking of migration/metastasis and invasion of tumor cells, and interfering with angiogenesis via receptor

tyrosine kinases. However, the detailed mechanisms of the anticancer properties of these secondary metabolites still remain unclear and not understand fully. Therefore, a comprehensive study about the mechanisms of action of these secondary metabolites should be carried out to evaluate their potential as novel remedies for treatment of different diseases.

2. Extensive literature research revealed that other sea cucumber has a long history as a traditional food and folk medicine. There is a great potential to utilize sea cucumbers to develop valuable functional foods with physiological benefits for human beings as ingredients of functional foods and nutraceuticals in the coastal zone of the Vietnam Sea.

NEW CONTRIBUTIONS OF THE THESIS

1. The present study is the first report on the chemical constituents and biological activities of the sea cucumbers *Stichopus horrens* và *Holothuria edulis* in the coastal zone of the Vietnam Sea.

2. From two sea cucumber species were isolated and identified 06 new compounds, including stichorrenosides A-E (*S. horrens*) and holothurin A_5 (*H. edulis*).

3. The aglycon part of stichorrenosides A-B containing the OH group at position C-22 and the Δ^7/Δ^{25} conjugated double bonds is a new structural aglycon. The 4-*O*-sodium sulphate-β-D-glucopyranose sugar unit in stichorrenoside B and the OOH group at position C-25 was first reported holothurin A_5 as a triterpene glycoside isolated from sea cucumber at the time of publication.

4. The present study is the first report to evaluate the ability of deacetylstichloroside C_1 to kill cancer cells according to the mechanism of action on biological targets. The results showed that this compound affects the cell cycle in the sub-G_1 phase and induces MCF7 cell apoptosis.

PUBLICATIONS WITHIN THE SCOPE OF THESIS

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