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LC-HRMS Based Approach for Identification and Quantification Analysis of Chemical Constituents of Sea Cucumbers from Aegean Sea - Their Cytotoxic and Antiviral Potentials

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Abstract: There are nearly 1200 species of sea cucumber in the world's seas. Among these creatures included in the Holothuroidea class, 37 species show distribution in the Mediterranean and Aegean Sea. The purpose of this study is to determine the chemical content and biological potent of five sea cucumber species, *Holothuria tubulosa*, *Holothuria poli*, *Holothuria mammata*, *Holothuria sanctori* and *Stichopus regalis* which were collected from the Aegean Sea. The detailed flavonoid, phenolic and triterpene contents were determined by LC-HR/MS. Cytotoxic activities against several cancer cell lines, MDA-MB-231, PC-3, A549, PANC-1, HEPG2 and a healthy cell line CCD-34LU were performed by MTT method. Antiviral activities of the samples were measured as virucidal activity against avian coronavirus by *in ovo*. According to the results of LC-HRMS analysis, *H. sanctori*, *H. poli* and *S. regalis* had the richest chemical content diversity in terms of examined triterpene compounds. Fumaric acid was detected as the most abundant substance in all sea cucumber species. *H. tubulosa* had a highly toxic effect on all the tested cells. The best cytotoxic activity on A549 cells was seen in *H. mammata*, *H. sanctori* and *H. poli*. *H. sanctori* also showed a significant toxic effect against PANC-1, MDA-MB-231, HepG2 and A549 cells, whereas the IC_{50} value in CCD-34LU cells was above 50 µg/mL for this sample. The *n*-butanol extracts of sea cucumber species reduced hemagglutination (HA) virus titer between 1-fold to 4-fold in log2-based at all tested concentrations. The best inhibited virus HA titer results were found in *H. tubulosa* at 5 μg/g. According to these results we have obtained, the extracts of sea cucumbers may be used in many fields such as medicine, food, cosmetics in the future. This study is also very important in terms of being a guide for all studies on the use, processing and production of sea cucumbers and detailed isolation and purification studies on sea cucumber species from Turkiye.

Keywords: *Holothuria; Stichopus;* cytotoxicity; antiviral activity; LC-HRMS; avian coronavirus.

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1. Introduction

Natural products are major source of compounds with extensive structural diversity and numerous therapeutic activities. One of the richest natural product source in the world is marine organisms. Natural compounds and their derivatives obtained from marine organisms find usage in the treatment of various diseases and in many industrial areas [1]. Sea cucumbers, one of the marine organism, are invertebrates of the Holothuroidea class of echinoderms. Their bodies are elongated in the direction of the axis passing from the anus to the mouth and resemble a sausage or cucumber [2]. There are approximately 1200 known sea cucumber species in the world oceans [3] while more than 70 species of them are commercially exploited [4]. They widely distributed across in Aegean coasts of Turkiye [5-8]. The sea cucumber species are considered as a potential source of dietary supply which has many pharmacological effects such as aphrodisiacs, anti-HIV and anti-cancer [9-12]. They have also various traditional usage as a tonic in Chinese and Malaysian literatures for their effectiveness against hypertension, asthma, rheumatism, cuts, burns, impotence and constipation [13,14]. These therapeutic properties and medicinal benefits of sea cucumbers can be attributed to the presence of considerable amounts of bioactive compounds, especially triterpene glycosides [15,16], phenolic, flavonoid compounds [17], polysaccharides [12], sterols, lipids and fatty acids [18].

As far as we know, previously no comprehensive article has ever been published covering the detailed triterpene, phenolic, flavonoid contents and cytotoxic, antiviral potentials of Turkish sea cucumbers. Triterpene compounds are the most important and abundant secondary metabolites in sea cucumbers. The biological function and the membrane properties of sea cucumbers are indicative of their role in external defensive action due to their triterpene content. Many literatures showed that high amounts of triterpene compounds in sea cucumber extract could have virocidal and cytotoxic effects [9, 15-16]. In this circumstances, the main purpose of our study is to determine the biological activity potentials and detailed phytochemical contents of 5 different sea cucumber species (*H. tubulosa*, *H. poli*, *H. mammata*, *H. sanctori* and *Stichopus regalis*) in our country, for the first time. Flavonoid, phenolic and triterpene profile of these species were evaluated by LC-HRMS. Cytotoxicity of *n*-butanol extracts were screened against several cancer cell lines, namely MDA-MB-231, PC-3, A549, PANC-1, HEPG2 and a healthy cell line CCD-34LU by MTT assay. Antiviral activities were also measured as virucidal activity against avian coronavirus by *in ovo*.

2. Materials and Methods

2.1. Sea Cucumber Materials

Holothuria tubulosa, Holothuria poli, Holothuria sanctori, Holothuria mammata and *Stichopus regalis* are all member of Holothuroidea class and spread out especially in Mediterranean and Aegean Sea coasts. Sea cucumber samples were collected by bottom trawl fishing technique from Ildir Gulf, Cesme coasts of Izmir city of Turkiye (*H. tubulosa, H. poli, H. sanctori, H. mammata*: 38°39'61.10″ N, 26°46'96.17″ E; *S. regalis:* 38°40'61.10″ N, 26°44'76.18″ E) (Figure S1, see supporting information). After the species were defined morphologically, the length and weight measurements of the individuals were made according to literature knowledge (Figure 1) [19, 20]. The exact length and weight measurements were also given in Table 1. Abdominal parts of all species measured were cut from anterior to posterior by a scalpel. Then, internal organs were cleaned and pairs of five longitudinal muscular bands of *S. regalis* species were removed with a scalpel (Figure 2f). Samples homogenized and stored -20 °C until analyses.

2.2. Chemicals

100 mg/L dihydrocapsaicin (97%, Sigma-Aldrich) solution was freshly prepared as stock solution was used as an internal standard (IS). Following compounds were used as standards for method validation in LC-HRMS analysis: Ascorbic acid (≥99%, Sigma-Aldrich), chlorogenic acid

(≥95% Sigma-Aldrich), fumaric acid (≥99% Sigma-Aldrich), orientin (>97% TRC Canada), caffeic acid (≥98% Sigma-Aldrich), caffeine (≥99 % Sigma-Aldrich), luteolin-7-rutinoside (>97% Carbosynth limited), vanilic acid (≥97% Sigma-Aldrich), luteolin-7-glucoside (>97% TRC Canada), rosmarinic acid (≥96% Sigma-Aldrich), hyperoside (>97% TRC Canada), apigenin-7-glucoside (>97% EDQM CS), salicylic acid (≥98% Sigma-Aldrich), naringenin (≥95% Sigma-Aldrich), luteolin (95% Sigma-Aldrich), nepetin (98% Supelco), apigenin (>97% TRC Canada), (-)-sinensetin (>97%, TRC Canada), chrysin (≥96% Sigma-Aldrich), caffeic acid phenethyl ester (CAPE, ≥97%, Sigma-Aldrich). Oleanane type triterpenes were isolated from our previous works as pomolic acid (>97%), tormentic acid (>97%) [21-23], aristatoside C (>93.7%) [24], cephoside B (>99.1%) [25], balansoid A (>99.3%) [26], dipsacoside B (>99.2%) [24,27], scoposide B (>99.9%) [28], tchihatchewoside A (>99.1%), tchihatchewoside B (>99.7%) [29], macranthoside A (>98.6%) [24,30], saponin A [3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosylhederagenin 28-*O*-*β*-D-glucopyranosyl ester] (>99.7%) [21], saponin B [3-O-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosylhederagenin 28-O-*β*-D-glucopyranosyl ester] (>99.9%) [21], dipsacus saponin A (>99.1%) [31,32].

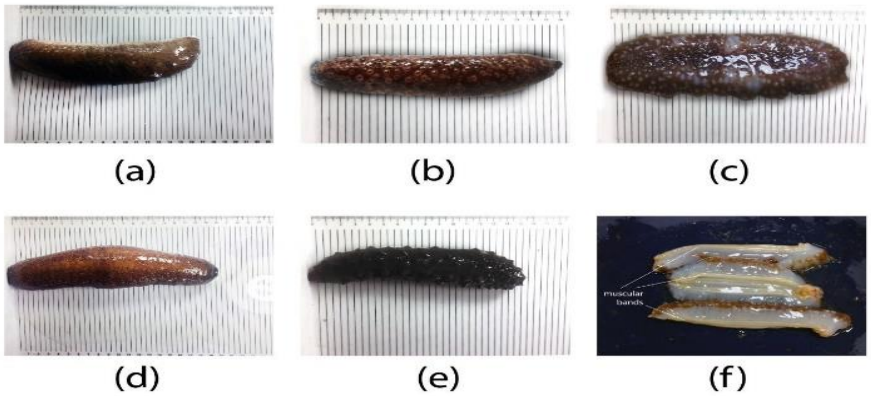

Figure 1. Sea cucumbers species (a) *H. tubulosa* (b) *H. sanctori* (c) *S. regalis* (d) *H. poli* (e) *H. mammata* and (f) muscular bands of *S. regalis*.

Table 1. Lengths and weights of sea cucumbers (n=5, mean ±SD)

Species	Length (cm)	Weight (g)
H. tubulosa	18.12 ± 1.39	126.26 ± 24.75
H. poli	17.92 ± 1.45	126.04 ± 26.11
H. mammata	17.66 ± 1.84	129.92 ± 25.39
H. sanctori	17.80 ± 1.53	127.62 ± 20.02
S. regalis	18.46 ± 1.07	130.24 ± 26.24

2.3. Preparation of Extracts

After collecting all sea cucumber species, they were stored at -20 ºC until the extraction process. The drawn samples were extracted three times with methanol (10 mL each) with a laboratory homogenizer (Silverson, L5M-A, USA) for 3h at room temperature. The extracts were concentrated under reduced pressure to dryness at 40 ºC. And then, MeOH residues were extracted three times with *n*-BuOH:H_2O (1:1) solvent system (each 5 mL). After separation of the *n*-BuOH and H_2O fractions, the *n*-BuOH fractions containing biologically active components were concentrated under reduced pressure. All extracts were kept on dark at +4°C until LC-HRMS analysis and biological activity studies.

2.4. Preparation of samples for LC-HRMS Analysis and Optimization of the Method

The dried 50-100 mg of the extracts of each species were dissolved in water:methanol (40:60) in a 5 mL volumetric flask. The flask was kept in an ultrasonic bath until a clear solution was obtained. Then, 100 μL of dihydrocapsaicin solution (from 100 ppm stock solution) was added as an internal standard and diluted to the volume with mobile phase and mixed and warmly heated to get clear solution. The solution was filtered through a 0.45 μm Millipore Millex-HV filter and the concentration of final solution (1 mL) was transferred into a capped auto sampler vial, from which 2 μL of sample was injected to LC for each run. The samples in the auto sampler were kept at 15^{o}C during the experiment [33-36].

LC-HRMS experiments were achieved on a Thermo ORBITRAP Q-EXACTIVE mass spectrometry equipped with a Troyasil C18 column (150 x 3 mm i.d., 3 μm particle size) for phenolic and triterpene saponin measurements. Two different mobile phase system were used herein. For simple compounds and phenolics measurements, the mobile phases A and B were composed of 1% formic acid-water and 1% formic acid-methanol, respectively. While the gradient programme of which was 0-1.00 min 50% A and 50% B, 1.01-6.00 min 100% B, and finally 6.01-10 min 50% A and 50% B. For triterpene saponins, gradient program of which was started 50% A and 50% B, 0.01-1 min, 75% B, 1.01-3.00 min 85% B, 3.01 and 15.00 90 %B, and finally 15.01-25 min 50% A and 50% B with the same mobile phases [29]. The flow rate of the mobile phase was 0.35 mL/min, and the column temperature was set to 22°C. Environmental conditions were set as temperature 22.0 ± 5.0 °C and relative humidity (50 ± 15) % rh [29,35]. The best mobile phase was determined to be an acidified methanol and water gradient in HPLC method. This mobile phase was also found to be suitable for ionization abundance and separation of compounds. The best ionization of small and relatively polar compounds was obtained by ESI source. The ions between m/z 85-1500 were scanned in high resolution mode of instrument [29,34,37-39]. Identification of compounds was done by comparison of retention time and HRMS data of standard compounds (in the range of purity 95%-99% see section chemicals). Dihydrocapsaicin (purity 95%) used as an internal standard for LC-HRMS measurements in order to reduce to repeatability problem of caused by external effects, such as ionization repeatability, in mass spectrometry measurements. The detailed mass parameter of each target compound was given in Supplementary Information in Table S1.

2.5. Chemicals and Cell Culture Materials

For cytotoxicity assay, healthy human lung fibroblast cell line (CCD-34LU), human prostate adenocarcinoma cell lines (PC-3), human pancreas epithelioid carcinoma cell lines (PANC-1), human breast adenocarcinoma cell lines (MDA-MB-231), human liver hepatocellular carcinoma cell lines (HepG2), human alveolar adenocarcinoma cell lines (A549) were purchased from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco, NY, USA). For antiviral activity assay, specific pathogen-free embryonated chicken eggs (SPF-ECE) and 1% chicken RBC (red blood cell) purchased from Bornova Veterinary Control Institute in Izmir-Turkiye.

2.6. In vitro Cytotoxicity Assay

In vitro cytotoxicity of sea cucumber samples was assessed on CCD-34LU, PC-3, PANC-1, MDA-MB-231, HepG2, A549 cells by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)] (ACROS, NY, USA) assay based on cell viability [40]. The principle of this assay is to cleavage of MTT that forms formazan crystals by cellular

succinate-dehydrogenases in viable cells. For this purpose, all cells which have $1x10^5$ cells/mL initial concentration were cultivated for 24 hours in 96-well microplates at 37 °C in an atmosphere humidified with 5% CO_2. Sea cucumber samples at the different concentrations (50, 5 and 0.5 μg/mL) were added to the cells and incubated for further 48 hours at 37 °C. Doxorubicin was used as positive control. The optical density of dissolved formazan crystals in DMSO were measured at 570 nm with the help of UV–Visible spectrophotometer. The percentage of viable cells were calculated with the below formula. Percentages of viable cells for each sea cucumber sample were determined with the below formula.

$$\text{Viable cells\%} = \frac{(\text{absorbance of treated cells}) - (\text{absorbance of blank})}{(\text{absorbance of control}) - (\text{absorbance of blank})} \text{ x } 100$$

The half maximal inhibitory concentrations (IC_{50}) were calculated by GraphPad Prism 5

2.7. In ovo Antiviral Activity Assay

2.7.1. Preparation of Virus

The avian coronavirus infectious bronchitis virus (IBV) strain D274, which is known as the European IB strain and originated in the Netherlands [41], was kindly presented for our experiments by Dr. Fethiye Coven (Izmir Bornova Veterinary Control Institute, Turkiye). Embryos were used before day 16 (or earlier) of incubation [42]. It was used to inoculate SPF-ECE's. The embryo infectious dose (EID50) of the IBV propagated from chorioallantoic fluid (CAF) was measured using the method defined by Reed and Muench (1938) [43]. The 100 EID50/0.1 mL stock virus solution was prepared by diluting phosphate-buffered saline (PBS) and used in this experiment. The stock virus solution was stored at -86 °C freezer. It did not require any cryoprotective agent during storage due to the properties of CAF.

2.7.2. Inoculation of sample-virus mixture

Antiviral activities of the samples were measured as virucidal effect [44]. Samples were diluted to final concentration (0.01, 1 and 5 µg/g) using PBS. Favipiravir was used as an antiviral agent and dissolved in DMSO-ultrapure water (1:9). Favipiravir final concentration was administered containing less than 5% DMSO. The diluted concentrations of samples and control groups were combined with the stock virus at a 1:1 ratio. Then the 0.1 mL of mixtures incubated for 1-hour at room temperature were injected to 9-11-day-old and SPF-ECEs were incubated at 37°C for 48 h. The viability of SPF-ECEs was recorded daily in order to conduct HA assays, CAF from SPF-ECE's were collected [44].

2.7.3. HA Assay

HA assay was applied to determine the virus titer using two-fold dilutions of CAF as described in the OIE protocol (2018) [44]. V-bottom 96-well microplates were used for HA assay and dilution was made by PBS. The wells were examined for button shape formation, which was determined by visually inspecting the endpoint of the dilution series. Visual examination of the endpoint was interpreted [45,46]. HA assay protocol to test antiviral activity was approved by Ege University, Local Ethical Committee of Animal Experiment (Date: 2020 No: 2020-051).

3. Results

In this study, a total of thirty-one phenolic and triterpene compounds were quantitatively determined by LC-HRMS in the n-butanol extracts of five different sea cucumbers (*H. tubulosa, H. poli, H. sanctori, H. mammata, S. regalis*). The body wall and muscular bands of *S. regalis* were also examined separately. Table 2 shows the phenolic and triterpene compounds content of (mg/kg) sea cucumber extracts. According to the results, the total number of detected compounds were detected as 19, 28, 19, 23, 21, 27 compounds in *H. tubulosa, H. poli, H. mammata, H. sanctori, S. regalis* and muscular bands of *S. regalis*, respectively.

Table 2. Phenolic and triterpene content of (mg/kg extract) sea cucumber species from Aegean Sea

Compounds	*H. tubulosa*	*H. poli*	*H. mammata*	*H. sanctori*	*S. regalis*	Muscular bands of *S. regalis*	U%
Ascorbic acid	27.35	50.10	98.74	21.16	132.86	27.93	3.94
Chlorogenic acid	0.05	6.10	3.42	0.78	7.07	9.62	3.58
Fumaric acid	117.95	177.40	1228.62	180.39	1070.79	168.71	2.88
Orientin	1.65	6.40	3.20	1.12	10.29	10.81	3.67
Caffeic acid	<LOD	0.60	0.36	0.30	0.86	2.03	3.74
Caffeine	6.60	5.30	35.75	4.07	4.39	0.88	3.06
Luteolin-7-rutinoside	<LOD	0.20	<LOD	<LOD	0.11	0.75	3.06
Vanilic acid	9.95	13.30	<LOD	3.53	<LOD	10.90	3.49
Luteolin-7-glucoside	0.45	1.00	0.95	0.45	2.68	2.96	4.14
Hyperoside	<LOD	0.60	<LOD	<LOD	<LOD	0.93	3.46
Apigenin-7-glucoside	<LOD	0.20	0.07	0.03	0.54	0.75	3.59
Salicylic acid	6.40	7.00	11.58	6.16	5.25	2.21	1.89
Naringenin	4.50	6.50	5.10	2.51	9.86	3.18	4.20
Luteolin	0.15	0.20	0.15	0.09	0.32	0.79	3.42
Nepetin	0.05	0.10	0.07	<LOD	<LOD	0.04	2.19
Apigenin	<LOD	0.30	0.15	<LOD	0.64	0.49	2.87
Sinensetin	0.10	0.20	0.07	0.03	0.11	0.09	3.36
CAPE	0.05	0.10	<LOD	<LOD	0.11	<LOD	3.13
Chrysin	1.55	2.60	0.73	0.39	1.71	0.62	3.24
Cephoside B	0.50	2.60	82.06	101.80	2.14	1.37	11.21
Dipsacoside B	<LOD	23.10	<LOD	5.31	<LOD	<LOD	3.51
Saponin A	5.45	3.70	<LOD	<LOD	15.11	2.96	4.29
Scoposide B	<LOD	<LOD	<LOD	0.06	<LOD	0.09	0.96
Saponin B	<LOD	0.90	<LOD	0.63	0.21	0.35	9.82
Aristatoside C	<LOD	<LOD	<LOD	0.66	<LOD	0.31	4.60
Tormentic acid	43.70	43.60	<LOD	57.37	205.50	68.82	3.54
Balansoid A	<LOD	<LOD	<LOD	0.36	<LOD	<LOD	4.52
Macranthoside A	<LOD	1.80	1.17	<LOD	<LOD	<LOD	11.10
Dipsacus saponin A	0.40	0.90	7.50	1.72	<LOD	10.50	6.74
Pomolic acid	32.45	33.00	<LOD	34.10	96.86	32.80	1.68

Fumaric acid was the most abundant substance which was detected between 117.95 and 1228.62 mg/kg extract in all sea cucumber species. Due to its non-toxic nature, fumaric acid is used in many fields from chemistry to food. According to actual literatures, fumaric acid demand increased by 46.5% between 2012 and 2020 years [47]. Most of the fumaric acid needs are met by petroleum-based syntheses, and due to limited petroleum resources, research interest has focused on obtaining fumaric acid from renewable and biological sources [48]. This study showed that sea cucumbers can be a good renewable biosource of fumaric acid.

This study also proved that these sea cucumber species contain 2 triterpene aglycones (tormentic and pomolic acids), and 9 saponins (cephoside B, dipsacoside B, saponin A, scoposide B, saponin B, aristoside C, balansoid A, macranthoside A, dipsacus saponin A) which has not been examined until

today. *H. sanctori, H. poli* and *S. regalis* had the richest chemical content diversity in terms of examined saponins. While muscular bands of *S. regalis* included eight triterpenes, the body wall of *S. regalis* contained only four triterpenes, cephoside B, saponin A, saponin B and tormentic acid. Besides that, triterpene amount of two different parts of *S. regalis* were showed difference. The most obvious difference in different parts of *S. regalis* was seen in the content of pomolic and tormentic acids. While the tormentic and pomolic acids contents in *S. regalis* muscle part were detected as 68.82 and 32.80 mg/kg, in *S. regalis* body wall was determined as 205.50 and 96.86 mg/kg, respectively. Cephoside B, a triterpene saponin compound, was found in all studied species. Dipcacus saponin A was also detected in all extracts except *S. regalis* wall. Aristatoside C, one of the biologicallly active saponin, was measured in the *S. regalis* muscle band and *H. sanctori* as 0.31 and 0.66 mg/kg, respectively. In our previous studies, it has been proven that aristatoside C had a cytotoxic effect on lung cancer cells after 48 hours of incubation and had a considerable cytotoxicity potential at lower doses compared to a chemotherapeutic agent doxorubucine [49]. In this respect, detection of aristatoside C in good amounts is very important finding

Table 3. IC_{50} values of *S. regalis, H. sanctori, H. tubulosa, H. mammata, H. poli*, muscular bands of S. *regalis*, doxorubicin against different cell lines (μg/mL)

Samples	A549	CCD 34LU	HEPG2	MDA MB	PANC-1	PC-3
S. regalis	46.46 ± 10.25	>50	>50	>50	>50	>50
H. sanctori	4.20 ± 0.29	>50	30.28 ± 7.23	28.52 ± 6.53	24.79 ± 3.71	>50
H. tubulosa	7.08 ± 1.21	17.75 ± 0.17	15.48 ± 0.83	18.14 ± 1.16	19.57 ± 3.18	11.47 ± 1.78
H. mammata	1.37 ± 3.70	>50	>50	>50	35.12 ± 0.33	>50
H. poli	4.05 ± 1.75	11.17 ± 1.39	>50	17.80 ± 3.48	7.13 ± 2.79	16.46 ± 3.66
Muscular bands of *S. regalis*	11.13 ± 5.94	>50	13.98 ± 0.36	>50	>50	>50
Doxorubicin	13.32 ± 1.40	2.37 ± 0.43	9.05 ± 0.33	11.77 ± 1.21	5.59 ± 1.49	4.45 ± 1.34

Cytotoxicity assays were tested by MTT analysis on a healthy cell line (CD-34LU) and a variety of cancerous cell lines (PC-3, PANC-1, MDA-MB-231, HepG2, A549) for sea cucumber samples (*S. regalis*, muscular band of *S. regalis*, *H. sanctori*, *H. tubulosa*, *H. mammata*, *H. poli*). Cytotoxic activity IC_{50} values and cell viability for the samples were shown in Table 3 and in Supplementary Information Figures S2-S7, respectively. IC_{50} values for all sea cucumber extracts were calculated in measurable range (0.5-50 μg/mL) for A549 cells. *H. mammata* and *H. poli* exhibited significant cytotoxicity against HepG2 cells PANC-1 cells, correspondingly with IC_{50} values of 5.28±0.13, 7.14±2.80 μg/mL, respectively which was more potent than the doxorubicin as a positive control. In addition, the IC_{50} value of *H. tubulosa* extract in PANC-1 cells was found to be similar to doxorubicin (9.58±0.39 μg/mL). For this cell line, *H. sanctori, H. tubulosa* and *H. poli* extracts exhibited the highest cytotoxic activity (4.30±0.29, 7.09±1.22 and 4.06±1.76 μg/mL, respectively). *H. tubulosa* and *H. poli* samples showed a cytotoxic effect against all cells (IC_{50}<50). In contrast, no significant cytotoxic effects were observed in the wall and muscular band of *S. regalis* for CCD-34LU, PC-3, PANC-1 and MDA-MB-231 cells.

The antiviral effects of sea cucumber samples (*S. regalis, H. sanctori, H. tubulosa, H. mammata, H. poli*) were assessed *in ovo* against IBV [41,44]. Samples incubated with 100EID50 IBV suspension for 1-hour were inoculated into SPF-ECE's. At the end of 48-h incubation of the samples,

embryos were determined for all groups and egg deaths were recorded daily by controlling embryo viability (Supplementary Information Figures S8-S9). In the virus control group and vehicle control group which contain 5% DMSO, there was no death on embryos. On the other hand, it was not recorded any death in the untreated control. Favipiravir as the positive antiviral drug was tested at two different concentrations (10 and 25 μg/g) and embryo mortality was found to be 0%. The positive antiviral control, favipiravir evaluated at 10 and 25 μg/g reduced the HA titer as 3-4 log2 HA titers in dose-dependent manner at 10 µg/g and 25 µg/g, respectively. In log2-based HA analysis, it was determined that *n*-butanol extracts of different sea cucumber samples at different concentrations reduced HA titer in same ratio (between 1-4 log2 HA). Among samples, *H. tubulosa* at 5 μg/g exhibit the highest HA titer. The decrease in sea cucumber concentrations resulted in a decrease in mortality (Table 4). Sea cucumber samples had high mortality rates and toxic effects on embryos in a dose-dependent manner.

Table 4. The virucidal antiviral effects of *S. regalis, H. sanctori, H. tubulosa, H. mammata, H. poli* on IBV after 48h incubation

Samples	Conc. (μg/g)	Egg mortality	% Mortality	HA titer	HA titer (log2)
Untreated SPF-ECE control		0/4	0	0	0
Only virus control		0/4	0	2048	11
Vehicle control (Virus treated with 5% DMSO)		0/4	0	2048	11
Favipiravir	10	0/4	0	512	9
(Antiviral drug agent)	25	0/4	0	256	8
S. regalis	0.01	0/4	0	512	9
	1	1/4	25	1024	10
	5	2/4	50	1024	10
H. sanctori	0.01	0/4	0	512	9
	1	2/4	50	512	9
	5	3/4	75	256	8
H. tubulosa	0.01	1/4	25	256	8
	1	1/4	25	256	8
	5	2/4	50	128	7
H. mammata	0.01	0/4	0	512	9
	1	1/4	25	512	9
	5	3/4	75	512	9
H. poli	0.01	1/4	25	256	8
	1	1/4	25	512	9
	5	3/4	75	1024	10

4. Discussion

The growing interest to sea cucumbers makes researchers increasingly curious about the chemical constituents and biological activities of them. Studies also conducted to date the rich chemical content of sea cucumbers containing biologically active compounds such as mainly saponins, glycosamines, sulfated polysaccharides, peptides, phenolics, sterols, cerebrosides, fatty acids and carotenoids. Sea cucumbers have the highest saponin content among marine organisms. Holothurian saponins are generally very specific due to the marine environment and most have triterpene aglycone with lactone skeleton called holostane. In recent studies, saponin scanning, isolation, characterization and activity studies were carried out in various sea cucumber species [16,50,51].

A number of studies have focused on the cytotoxic activity of different types of sea cucumber extracts. Alper and Gunes (2020) [17] examined activities of *H. tubulosa* using MTT method against A549, HeLa, PC-3, MCF-7 human cancer cell lines and HEK293 and BEAS-2B normal cell lines. In

that study, in which aqueous and methanol extracts were examined, water extract was observed to have lower IC_{50} values in all cell lines. On the other hand, methanol extract showed activity against only A549 and HeLa, depending on different time intervals. HEPG2 and PANC-1 cell lines have not been studied for *H. tubulosa*. In our study, *n*-butanol extracts were evaluated against cancer lines PC-3, PANC-1, MDA-MB-231, HepG2, A549 and a healthy cell line CCD-34LU similar to this study and it was found that *n*-butanol extract of *H. tubulosa* showed the highest cytotoxic activity against A549 cells. Another cytotoxic activity study was performed on *H. poli* by Omran and Khedr (2015) [52]. The study demonstrated the efficacy of *H. poli* as a cytotoxic agent against two tumor cell lines, HCT116 (colon adenocarcinoma cell line) and MCF7 (breast adenocarcinoma cell line). According to the literature reviews, no studies were conducted against cell lines (PC-3, PANC-1, MDA-MB-231, HepG2, A549 and a healthy cell line CCD-34LU cells) determined on *H. poli*, *H. sanctori*, *H. mammata* and *S. regalis*. In our study, the strongest cytotoxic activity was determined in *H. mammata*, *H. sanctori* and *H. poli* samples on A549 cells. This suggests that the presence of aristatoside C, which has very high activity against A549 cells in *H. sanctori*, may be the source of cytotoxicity [24]. In another study, the cytotoxicity of the three different sea cucumber extracts was evaluated against A549 and B16F10 cells. Among these species, *H. atra* has shown the highest toxicity. IC_{50} values were found as 1.8-20.9 μg/mL for A549 cells and 0.5-85.0 μg/mL for B16F10 cell [53]. In a different study, methanol extracts of 15 sea cucumber species were examined their cytotoxicity in T47D cells. Among them, *H. atra* and *H. edulis* were reported the best cytotoxic values at 23.00 and 54.21 μg/mL, respectively. No cytotoxic effects have been observed in other extracts [54]. The results of this study have been found similar to the other studies stated in the literature. As a result of this study, it was significant result that *H. sanctori* showed cytotoxic effects on PANC-1, MDA-MB-231, HepG2 and A549 cells while it had not cytotoxicity in healthy cells, CCD-34LU. Different from *H. sanctori* sample, it has been observed that *H. tubulosa* and *H. poli* had cytotoxicity at very low concentrations, but they have showed the same effect in the CCD-34LU cells. Depending on the content and biological activity of the extracts of sea cucumbers, different cytotoxicity values have been found. The regions where samples were collected and the environmental conditions in those regions could have been effective in the observation of different cytotoxic values.

Sea cucumbers were researched as antiviral efficacy as well as in many therapeutic areas. Various studies have been assessed the antiviral activity of sea cucumbers *in vitro* cell culture. Huang et al. found that fucosylated chondroitin sulfates (FuCS-1) derived from sea cucumber can exert an inhibitory effect on HIV viral replication [53]. This compound prevents HIV-1 from entering cells [55]. In addition, Farshidpour et al. (2014) evaluated the antiviral activity of *Holothuria sp*. against herpes simplex virus type 1 (HSV-1). They found that polar extract had an antiviral effect against HSV-1 in cell culture [56,57]. Studies examining the *in ovo* antiviral activity of sea cucumber extracts are quite limited. In our study, evaluated the *in ovo* virucidal activity of sea cucumber samples against IBV in SPF-ECEs was evaluated. Many studies in the literature have reported that favipiravir is used as a broad-spectrum antiviral drug and inhibits the replication of different RNA viruses [56]. According to *in ovo* results in our laboratory conditions, it was found that favipiravir did not show strongest antiviral activity against IBV. This may be attributed to the fact that most antivirals are only effective against a particular virus. Comparing dose-dependent deaths, the sea cucumber samples tested in this experiment had a toxic effect on SPF-ECEs as the concentrations increased. According to the *in ovo* antiviral activity results, it is suggested that sea cucumber samples, which have a rich variety of triterpene compounds, exhibited antiviral activity. In addition to studies, extending the incubation time may help it to show better antiviral activity. The fumaric acid, the most abundant substance in all sea cucumber species, supported this antiviral activity, as well.

In conclusion, sea cucumber species has been one of the popular research topic in recent years. Market demand for them is increasing day by day, especially in the field of food and medicine [3, 58]. According to these results we have obtained, the extracts of sea cucumbers may be used in these fields such as food, medicine, cosmetics, etc. They have great potential to be used for dietary supplements, food additives, food preservatives and development of high value products for various industrial applications. This study is also very important in terms of being a guide for all studies on the use, processing and production of sea cucumbers and detailed isolation and purification studies on sea cucumber species from Turkiye.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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