



Cytotoxic Impacts of Holothurin B from Sea Cucumber *Holothuria tubulosa* via Inducing Apoptosis on Human Prostate and Pancreatic Cancer Cells

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Abstract

The sea cucumbers are recognized not only for their nutritional value but also for their therapeutic and pharmaceutical properties. Therefore, detailed chemical and biological studies were aimed on the sea cucumber species *H. tubulosa* which is common in the Aegean Sea, for the first time. Following extraction and chromatographic separation of the *n*-butanol extract, seven secondary metabolites were isolated one sterol sulfate, one stanol xyloside, two sphingolipid derivatives, and three saponins. Structural elucidation was achieved using advanced spectroscopic techniques, including 1D and 2D NMR. The cytotoxic and apoptotic effects of these compounds were evaluated via the MTT assay on CCD-34Lu, PC-3, PANC-1, A549, and U-87 MG cell lines. Among the compounds, holothurin B (5) exhibited the most potent cytotoxic and apoptosis-inducing activity, particularly in PC-3 and PANC-1 cells, with IC₅₀ values of 1.22±0.15 μM and 3.92±0.35 μM, respectively. Considering these results; the substance has great potential for planning further studies as a candidate saponin for use in cancer therapy. A drug formulation containing completely local and 100% natural saponin isolated from sea cucumbers growing in our seas can be considered as a robust potential for future *in vivo* and clinical research.

Introduction

Sea cucumbers (or holothurians) are marine echinoderms belonging to the class Holothuroidea. These creatures, whose habitats can be at different depths, are distributed in both shallow and deep seas, with more than 1,500 species worldwide (Pierrat et al. 2022). Commercially important sea cucumbers are distributed globally more than 70 countries worldwide. In Türkiye, among the 22 sea cucumber species identified along the Marmara, Aegean and Mediterranean coasts (Oztoprak 2014), the most economically valuable species are primarily *H. tubulosa*, *H. polii*, *H. mammata*, *Stichopus regalis* and *H. sancta* (Gunay et al. 2019; Dereli and Aydin 2021). 140 tons of these species were exported in 2023, with an economic

value of approximately 12.7 million dollars (TSI, 2023) and they are used for many purposes such as food industry, medicine, cosmetics and the pharmaceutical sector (Chen 2004; Kunili and Colakoglu 2018). Particularly in Asian cuisine, they hold a significant place, due to their rich nutritional value. They have also been used as a traditional medicine for centuries due to their therapeutic effects (Hamel et al. 2024; Hossain et al. 2022; Aminin et al. 2015). These marine creatures live in harsh conditions such as extreme temperatures, high pressures and salinity, and the lack of space, light and food in these conditions leads to intense competition among them (Williams et al. 1989). For this purpose, sea cucumbers produce toxins, which contain unique bioactive secondary metabolites, in order to survive and facilitate their adaptation to environmental conditions

use (Pangestuti and Arifin 2018). The toxin they secrete is rich in chemical content such as triterpene glycosides, peptides, sulfated polysaccharides, sterols and cerebrosides, have been the focus of numerous studies investigating their effects on cancer cells (Sarıkahya et al. 2022; Silchenko et al. 2005; Telahigue et al. 2020; Wargasetia et al. 2022). Many scientific researchers have proposed sea cucumbers as a rich source of bioactive compounds that possess significant apoptotic effects, cell cycle arrest, tumor growth reduction, antimetastatic and antiangiogenic effects, and drug resistance prevention (Wargasetia and Widodo 2017). Additionally, secondary metabolites isolated from sea cucumbers are capable of exhibiting numerous pharmacological activities, including antiangiogenic, anticoagulant, antihypertensive, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, and wound-healing activities (Bordbar et al. 2011; Khotimchenko 2018).

Triterpene glycosides are considered the primary toxicants in sea cucumbers and play a vital role in the defense of Holothuroidea against predators as toxins (Podolak et al. 2010; Osburn et al. 2011; Kim and Himaya 2012). Sea cucumber saponins have a different chemical structure specific to the marine environment compared to terrestrial plants, which plays an important role in the diversity of biological activities. Especially many studies illustrate that all these medical advantages are triterpene glycosides consisting of a hydrophobic 3 β -hydroxyholost-9(11)-ene aglycon, derived from lanostane with an 18(20)-lactone structure (Zhao et al. 2018). The lanostane type saponins comprised of one up to six sugar units, connected to position 3 of the sapogenin are also characteristic of sea cucumbers (Aminin et al. 2015). This makes them more special from the saponin aglycons reported in the plant kingdom (Puspitasari et al. 2022).

The current paper deals with the isolation, structure elucidation, cytotoxic activity and, apoptotic effects of seven bioactive compounds from *Holothuria tubulosa*, one of the most common and commercially significant species in the Aegean Sea (Befani et al. 2024; Rakaj et al. 2018). While crude extracts of this species have been studied previously, comprehensive isolation and detailed characterization studies remain limited. This is the first comprehensive study contain the cytotoxic and apoptosis effects of isolated and structural elucidated secondary metabolites from *H. tubulosa*. In this work, three known triterpene glycosides, one sterol sulfate, one stanol xyloside, and two sphingolipid derivatives were meticulously isolated and structurally elucidated from *H. tubulosa* collected in the Aegean Sea, for the first time. The purified compounds were tested for cytotoxic and apoptotic effects on human cell lines, including human lung fibroblast (CCD-34Lu), human prostate adenocarcinoma (PC-3), human pancreatic epithelioid carcinoma (PANC-1), human alveolar adenocarcinoma (A549) and glioblastoma (U-87 MG) using the MTT assay.

Materials and Methods

General

In the present study, a range of chromatographic techniques, suitable adsorbents, solvents, and instruments were employed through isolation, purification, and spectral analysis procedures. For the structural studies, the FTIR spectra and optical rotations were analyzed using a Mattson Genesis Series Fourier Transform Infrared Spectrophotometer and a Rudolph Research Analytical Autopol I Automatic Polarimeter, respectively. The 1D- and 2D-NMR spectra were recorded on a Varian AS 400 MHz and 600 MHz spectrometer in DMSO-*d*₆ with TMS as an internal standard. For the animal extraction procedure, Silverson AX5 emulsifier was used. Silica gel 60 (0.063–0.200 mm, Merck 7734), sephadex-LH 20 (25–100 μ m, Sigma-Aldrich) and lichroprep RP-18 (25–40 μ m, Merck 9303), were employed for column chromatography (CC) applications. For the open column chromatography procedures, Spectra/Chrom® CF-1 fraction collector was utilized for the collection of eluents at the same frequency. Thin layer chromatography (TLC) was performed on F254 (Merck 5554) and RP18 F254s (Merck 5560) pre-coated aluminum sheets. Samples and extracts were concentrated using a Buchi vacuum evaporator (Rota vapor RII, vacuum controller/V-850, vacuum pump/V-700). Dulbecco Modified Eagle Medium F-12 (DMEM/F12), Trypsin-EDTA, penicillin/streptomycin, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Gibco (USA). Doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (USA). The Invitrogen eBioscienceTM Annexin V Apoptosis Detection Kit was procured from Invitrogen (USA).

Echinoderm Material

A total of 100 *Holothuria tubulosa* was collected from İldır Bay (38°23'46.0" N, 26°28'10.6" E) located on the Cesme coast of Izmir, Türkiye by scuba diving at depths of 5–8 m in 2020 (Figure 1). Sea cucumbers were transported to Ege University Research and Application Center in Urla, Izmir and stored into 2 m³ (2×1×1m) capacity bare tanks without any sediment. Filtered natural seawater was used in the tanks with continuous water exchange from the adjacent sea. The sea cucumbers were removed from the sea water and kept on a dry sponge for 1 minute to remove external water, and then their weight was taken (Battaglene et al. 1999). After weighing procedure, digestive tracts were isolated. Abdominal parts of the samples were cut from anterior to posterior by a scalpel (Gunay et al. 2020). This study meets the ethical guidelines outlined by the American Fisheries Society.

Extraction Procedure

The body walls of fresh sea cucumbers (3.9391 kg) were dried under direct sunlight in August 2024 for 2 days. Due to its high water content, 88% loss by mass was calculated after drying. The dried materials (474.25 g) were grinded with the help of a mechanically laboratory grinder. Extraction was performed with MeOH (5 x 600 mL) via Silverson AX5 emulsifier for 6 hours at room temperature. The filtered extract solution was concentrated to dryness in vacuum at ~ 40 °C using a rotary evaporator. The dried extract (98.8564 g) was then extracted with *n*-BuOH:H₂O (1:1) solvent system (4 x 140 mL) using a separatory funnel. After extraction, organic and aqueous phases were separated. Both extracts were concentrated to dryness in vacuum at ~ 40 °C using a rotary evaporator and weighed, separately.

Isolation and Purification Processes

The dried *n*-BuOH extract (12.990 g) was chromatographed on silica gel open column chromatography (CC-1) using a stepwise gradient of hexane:ethyl acetate (90:10 \rightarrow 0:100%) as the mobile phase in the first stage of the purification and 32 fractions were obtained. The fractions were combined based on their TLC R_f values and subsequently re-chromatographed using suitable solvents and stationary phases. Fractions 10-11 (0.1900 g) was exposed to a silica gel open CC eluted with hexane:ethyl acetate (100:0 \rightarrow 0:100 %) yielded 7 fractions. The first

compound 1 (106 mg), was obtained from 5th fraction of this process. The combined fractions 16–18 (0.6248 g) of CC-1 were purified by Sephadex CC, utilizing molecular size differences elution with a dichloromethane:methanol (80:20 \rightarrow 0:100%) solvent system yielded 12 fractions. The compound 2 (73.8 mg) was isolated from the final fraction. Fraction 20 (0.0756 g) of CC-1 was successively subjected to silica gel CC and stepwise eluted with CHCl₃: MeOH: H₂O (90:10:0.5 \rightarrow 80:20:2), yielding seven fractions and 6th fraction afforded compound 3 (9.4 mg). Fractions 21-22 (0.2131 g) of CC-1 were subjected to another silica gel open CC and eluted with a CHCl₃:MeOH:H₂O (90:10:0.5 \rightarrow 80:20:2) to afford 8 fractions. Compound 4 (14.1 mg) was obtained in pure form from the 6th fraction (see *Supplementary Figure S3*). Fractions 24-27 (0.3670 g) of CC-1 were purified by silica gel open CC using CHCl₃: MeOH: H₂O (90:10:0.5 \rightarrow 80:20:2) as the eluent system, yielding 14 fractions and yielded compound 5 (75.6 mg) from fraction 8. The combined fractions 28-31 (1.4338 g) of CC-1 were subjected to silica gel open-column chromatography with CHCl₃: MeOH: H₂O (90:10:0.5 \rightarrow 70:30:3) and total of 14 fractions were collected. The compound 6 (12.8 mg) was obtained from combined fractions 10-12. The fractions 8-9 of this column were combined (0.2060 g) and further purified by reversed-phase silica CC resulted in 5 different fractions using the solvent system H₂O:MeOH (%100:0 \rightarrow %0:100). The fraction 2 was determined as compound 7 (57.1 mg) (Figure 2).

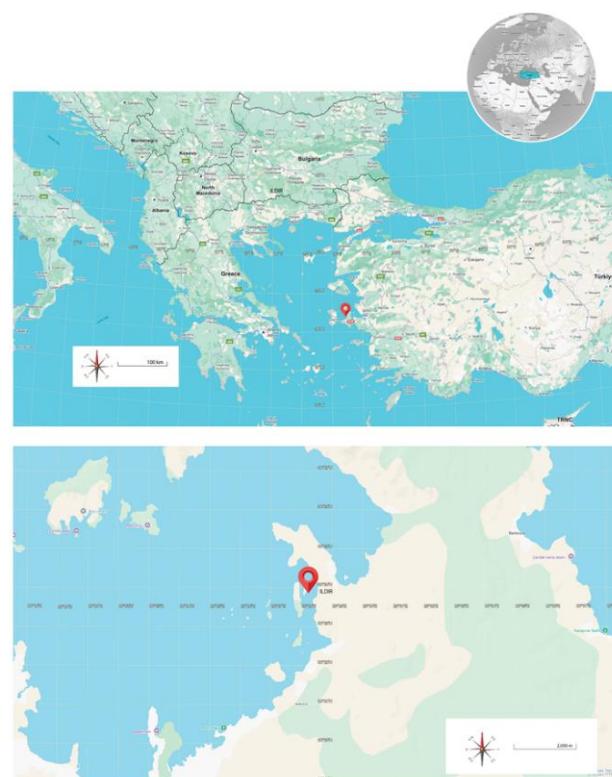


Figure 1. (a) Collecting spot and (b) the fresh material of *H. tubulosa*.

Cytotoxicity Assays

Cell Culture

A549 (human alveolar adenocarcinoma), PANC-1 (human pancreatic epithelioid carcinoma), PC-3 (human prostate adenocarcinoma), U-87 MG (glioblastoma), and CCD-34Lu (human lung fibroblast) were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cell lines were maintained in Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL of penicillin and 100 μ g/mL of streptomycin (Gibco). The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂. The cells were sub-cultured twice a week, and the cells in the exponential growth phase were used in the analysis.

In vitro Cytotoxicity Analysis

Cytotoxicity of the compounds was determined with MTT assay which is a colorimetric technique that detects the activity of succinate dehydrogenase (a mitochondrial enzyme for living cells) (Mossman 1983). Accordingly, A549, PANC-1, PC-3, U-87 MG, and CCD-

34Lu cells were seeded into 96-well microplates with an initial concentration of 1×10^5 cells/mL. Then, cells were incubated in a humidified environment for 24 hours at 37 °C. After incubation, cells were treated with different concentrations of saponin molecules (50, 5, and 0.5 μ g/mL) for 48 hours at 37 °C. Doxorubicin was used as the positive control (20, 2, and 0.2 μ g/mL). The treatment concentration was given in μ M for pure compounds. The optical density of formazan crystals was measured at 570 nm (reference filter, 690 nm) with UV visible spectrophotometer (Versamax Microplate Reader, USA). Cell viability (%) was calculated by the following formula:

$$\text{Cell Viability (\%)} = [(\text{Atc-Ab})/(\text{Ac-Ab})] \times 100$$

Where, Atc: Absorbance of treated cell, Ab: Absorbance of blank, Ac: Absorbance of control.

The IC₅₀ values were reported with $\pm 95\%$ confidence intervals (95% CI). The analysis was conducted utilizing the Graph Pad Prism 8 software. Morphological observations were made with an inverted microscope.

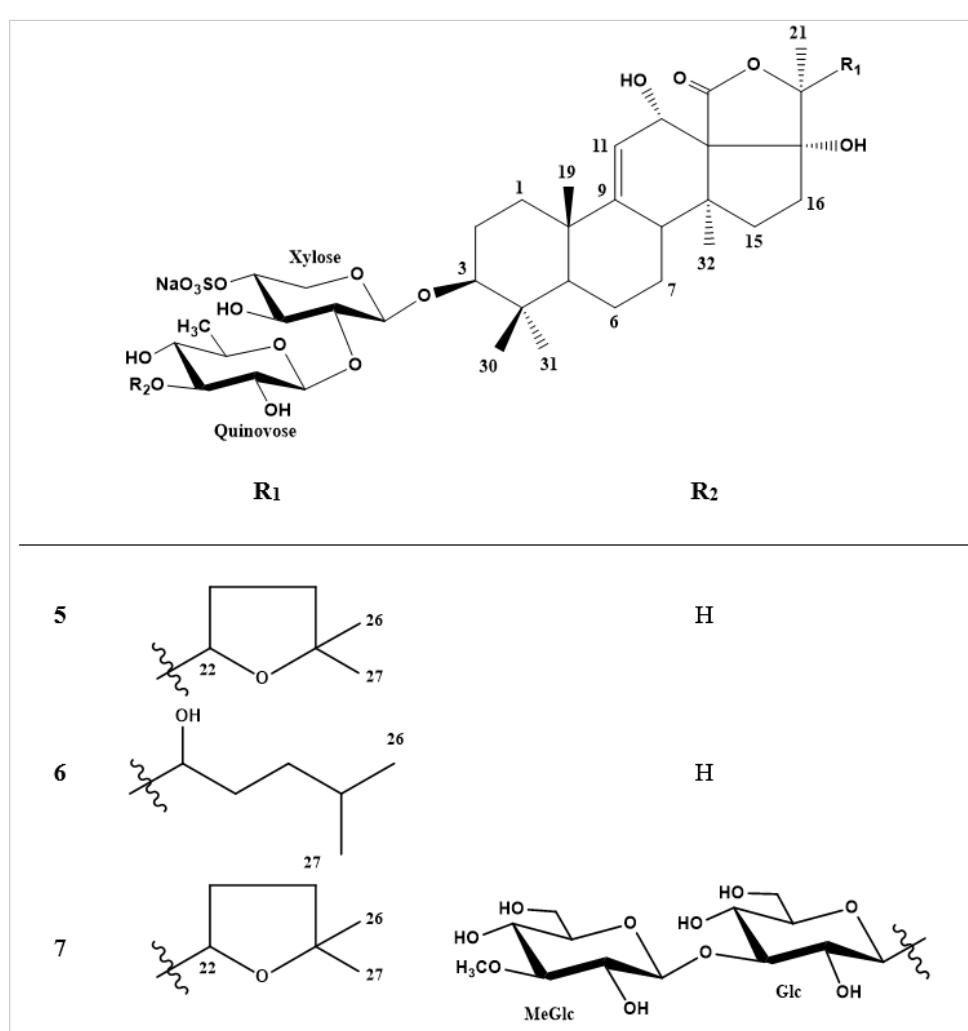


Figure 2. Chemical structures of isolated holothurin saponins (5-7).

Apoptosis Assay

Apoptosis, also known as programmed cell death, is typically distinguished by different morphological traits and energy-dependent molecular pathways (Zang et al. 2021). A flow cytometry-based Annexin V/Propidium Iodide (PI) assay was performed to evaluate apoptotic cell death. PC-3 and PANC-1 cells were seeded in 6-well plates at a density of 5×10^4 cells/mL and allowed to adhere overnight. Holothurin B saponin ($IC_{50}/2$, IC_{50} , and $2 \times IC_{50}$) was added to cells at different doses for a total of 48 hours. The cells were trypsinized and washed twice with PBS solution following the treatment period. To analyze apoptosis, the cells were resuspended in Annexin V binding buffer and stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (Invitrogen eBioscience™ Annexin V Apoptosis Detection Kit) and 15-minute dark incubation period was examined at room temperature for the staining process. Apoptotic populations were investigated using flow cytometry (BD Accuri 5) and data analysis was performed using FlowJo software to ascertain the proportions of necrotic (Annexin V⁻/PI⁺), late apoptotic (Annexin V⁺/PI⁺), and early apoptotic (Annexin V⁺/PI⁻) cells. All tests were repeated three times due to establish consistency and dependability (Karayildirim et al., 2021).

Statistical analysis

SPSS (Statistics Program for Social and Science) program version 23.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. The results of 3-replicate samples were compared with the variable groups and the control group, with mean \pm standard deviation values. One-way analysis of variance (ANOVA) was performed with GraphPad Prism 5 (USA) by Dunnett's test. $P < 0.05$ was considered significant.

Results

The presented investigation focused on the isolation, purification, and characterization of the chemical components, particular emphasis on sulphated triterpene glycosides from *Holothuria tubulosa* sea cucumber. According to our literature review, this is the first study conducted on isolation and purification of this species from Türkiye.

The average weight and average length of sea cucumbers were determined as 64.31 ± 16.6 g and 16.2 ± 2.55 cm, respectively, for the whole sampling (see Supplementary Figure S1 and S2). Following dried and ground echinoderm material underwent sequential extractions were completed with methanol, *n*-butanol and water. After various chromatographic procedures performed on the *n*-BuOH extract rich in saponins, a total of seven compounds, including one stanol xyloside, one sterol sulfate, two sphingolipids derivatives and,

three sulphated triterpene glycosides were isolated and purified. Advanced spectroscopic techniques, including 1D (1H , ^{13}C) and, 2D NMR (HMBC, HSQC, 1H -COSY) analysis, were used to elucidate the structures of the purified compounds. Compound 1 was identified as a cholestanol xyloside and was found to be a major component of the sea cucumber *Eupentacta fraudatrix*, while compound 2 was determined to be a sulfated sterol compound, specifically cholesterol sulfate which was isolated from *E. fraudatrix* (Makarieva et al. 1993). Compound 3 was characterized as sphingolipid derivative namely ceramide (Dabrowski et al. 1980) and was isolated previously from sea cucumber *Stichopus japonicas* (Yamada et al. 1999). Compound 4 was identified also as a sphingolipid derivative (Klenk 1942; Kuhn and Wiegandt 1963) namely ganglioside isolated from the sea cucumber *Cucumaria echinata* as one of the first study (Yamada et al. 1998) (Online Resource 1, see Supplementary Figure S3).

Compound 5 was identified as a sulphated triterpene glycoside named holothurin B, most abundant saponin, and first isolated from *H. vagabunda* and *H. lubrica* (Yasumoto et al. 1967). Compound 6 was identified as minor sulphated triterpene glycoside namely holothurin B₂ and firstly isolated from *H. polii* (Silchenko et al. 2005). The final isolated compound 7 was identified as sulphated triterpene glycoside called holothurin A, which is as abundant as holothurin B and elucidated the first time from sea cucumber *Actinopyga agassizi* (Chanley et al. 1960) (Figure 2). All the original NMR spectra and data of compounds 1-7 were presented in Figures S4-S32 and Tables S1-S2 in the Supplementary Materials, respectively.

The cytotoxic activity of the elucidated compounds 1-7 isolated from *H. tubulosa* was evaluated under *in vitro* conditions by MTT assay using healthy and several cancer cell lines (Table 1). The IC_{50} (half maximal inhibitory concentration) values of the compounds against cancer cells were calculated and compared to commercially available doxorubicin chemotherapeutic agent.

As illustrated in Table 1, (Online Resource 1, Figure S33 in the Supplementary) compound 1 showed selectively moderate cytotoxic activity compared to the other compounds across all tested cell lines against PANC-1 cells with an IC_{50} of 45.24 ± 1.66 μ M. It also exhibited an IC_{50} value of 40.44 ± 12.60 μ M in healthy CCD-34Lu cells, which is notably less potential than doxorubicin (23.46 ± 0.18 μ M), suggesting a weaker toxic effect on noncancerous cells. Compound 1 has also a lower effect compared to other compounds 5, 6 and 7 which are much more toxic on healthy cells. Compound 5 exhibited potential toxic effect against all the tested cell lines. Surprisingly, it was most potent in PC-3 and PANC-1 cells with IC_{50} values of 1.22 ± 0.15 μ M and 3.92 ± 0.35 μ M, respectively, identifying it as a potent anticancer drug in prostate and pancreatic cancer. To the best of our knowledge, holothurin B saponin is herein first tested in PC-3 and PANC-1 cells.

The compound 5 namely holothurin B had also cytotoxic effect, with IC_{50} values of $5.98 \pm 0.6 \mu M$ and $4.45 \pm 1.35 \mu M$, in U-87 MG and A549 cancer cell lines, respectively. Additionally, this compound was exhibited remarkably cytotoxicity in the normal fibroblast cell line CCD-34Lu with IC_{50} value ($5.02 \pm 1.19 \mu M$), indicating less toxicity toward non-cancerous cells. Compound 6 was found to have less effective cytotoxicity than holothurin B across in all examined lines. However, compound 6 was exhibited substantially lower cytotoxic effect on CCD-34Lu cells with IC_{50} values $19.88 \pm 1.06 \mu M$ when compared to holothurin B (compound 5). This finding suggests that compound 6 may not have cytotoxic effect on healthy cell line. The IC_{50} values of holothurin A (compound 7) were demonstrated as $40.64 \pm 6.41 \mu M$ in PANC-1 and $34.52 \pm 4.28 \mu M$ in PC-3 cells making it has the least cytotoxicity among other saponins. Furthermore, the IC_{50} values in U-87 MG and A549 lines of compound 7 were greater than $50 \mu M$, indicating weak efficacy or possible cell resistance. However, holothurin A exhibited the least cytotoxicity in healthy cells lines CCD-34Lu cells with IC_{50} value $24.56 \pm 2.38 \mu M$ among other saponins.

Additionally, the apoptotic effects of holothurin B saponin were assessed in PC-3 and PANC-1 cells, for the first time after staining the cells with FITC-conjugated Annexin V and analyzed by flow cytometry. The results showed that early apoptosis and late apoptosis were significantly induced in a dose-dependent manner in PC-3 cells. In PC-3 cells, the unstained and stained control groups were exhibited high viability with minimal apoptotic or necrotic activity. When the concentration of the compound increased, a dose-dependent induction of apoptosis was observed. A significant portion of cells entered late apoptosis with 48.8%, necrosis with 29.9% with and only 14.6% remaining viable at IC_{50} . The majority of cells underwent late apoptosis (76.8%), indicating strong cytotoxicity and pro-apoptotic effects at $2 \times IC_{50}$. Similarly, in PANC-1 cells, the control groups were exhibited high viability up to 99.5% with negligible apoptosis. The dramatic increase was observed with the treatment with $IC_{50}/2$ which resulted in late apoptosis (90.7%), while IC_{50} and $2 \times IC_{50}$ caused 68.9% and 82.0% late apoptosis, respectively. Across both cell lines, live cell populations decreased significantly in a dose-dependent manner.

While the necrotic cells ratio reached the highest value at $1.22 \mu M$ ($^{**}P < 0.01$) it was determined that the necrosis effect decreased at $2.44 \mu M$, ($^{*}P < 0.05$) and there was a shift towards apoptotic pathways. On the other hand, late apoptosis in PANC-1 cells was notably induced at all concentrations between $1.96-5.88 \mu M$ ($^{**}P < 0.01$, $^{***}P < 0.001$) (Figure 3, Figure S33). Unlike PC-3 cells, necrosis remained relatively low ($P > 0.05$), indicating that the compound 5 primarily induced apoptosis rather than necrotic cell death in PANC-1 cells. These findings suggest that the tested compound 5 (holothurin B) exerted the strong pro-apoptotic effects in both cancer cell lines, though the balance between apoptosis and necrosis varies depending on the cell type.

Discussion

Compound 1 has the similar aglycone unit as compound 2 but differs by containing a double bond (C22) and a methyl group (C24) in the side chain and a xyloside molecule attached to the 3rd carbon (see Online Resource 1, Supplementary Figure S3). The selective cytotoxicity of this saponin against PANC-1 cells can be explained by the balance of hydrophilic and hydrophobic characters formed by the number of sugars and the diversity of units in the side chain together with the aglycone (Ondevilla et al. 2023; Podolak et al. 2023). Although compounds 5, 6 and 7 belong to the same secondary metabolite class and show significant cytotoxic activity in all cell lines tested, IC_{50} values show significant differences among the compounds due to differences in both aglycone structure and sugar unit, indicating different cytotoxic potentials. This study also demonstrate that holothurin B, like other saponins frequently investigated in the literature, has the ability to interact with the cell membrane, disrupt the membrane and induce apoptosis. Moreover, holothurin B may exhibit selective cytotoxic effects against cancerous cells while being more protective of normal cells than other tested compounds. The increased cytotoxicity of holothurin B compared to doxorubicin suggests that it needs to be elucidated by *in vivo* studies and may have potential for further cancer research. These findings contribute to the promising cytotoxic potential of saponins obtained from sea cucumber and

Table 1 Determined IC_{50} values (μM) of compounds 1-7 via MTT assay against cancerous and healthy cell lines A549, PANC-1, PC-3, U-87 MG, and CCD-34Lu cells

	CCD-34Lu	PANC-1	CELL LINES		
			PC3	U-87 MG	A549
1	40.44 ± 12.60	45.24 ± 1.66	>50	>50	>50
2	>50	>50	>50	>50	>50
3	>50	>50	>50	>50	>50
4	>50	>50	>50	>50	>50
5	5.02 ± 1.19	3.92 ± 0.35	1.22 ± 0.15	5.98 ± 0.6	4.45 ± 1.35
6	19.88 ± 1.06	18.04 ± 8.08	11.12 ± 2.43	19.18 ± 4.95	28.82 ± 3.73
7	24.56 ± 2.38	40.64 ± 6.41	34.52 ± 4.28	>50	>50
Doxorubicin	23.46 ± 0.18	34.83 ± 1.36	8.67 ± 6.05	4.67 ± 1.66	21.56 ± 2.91

are consistent with previous studies that yielded similar findings (Aminin et al. 2015; Zhao et al. 2018). When the comparing of cytotoxicity of compounds 5 and 6 in all tested cell lines, noteworthy difference was observed. Although the sugar units in two compounds are completely same, it is clear that the aglycon part especially the side chain of holothurins was led to this difference (Figure 2). The tetrahydrofuran (THF) ring at C-20 of compound 5 was seemed to caused more effective cytotoxicity than aliphatic chain at C-20 of compound 6, which is obtained after tetrahydrofuran ring opening reaction. Compounds containing THF groups are generally lipophilic in character, so they are easier to transport through the cell membrane, increasing the cytotoxic effect (Wolfe et al. 2013). The major causes of high cytotoxicity in tetrahydrofuran ring bearing molecules are the generation of reactive metabolites, increased oxidative stress, DNA and protein binding potential, increased cell membrane transport, and systemic exposure (Mameri et al. 2021).

When apoptosis studies in the literature were examined, Pranweerapaiboon et al. (2021) reported that saponins obtained from *H. scabra*, one of the sea cucumber species, induced dose-dependent apoptosis

in human cancer cell lines, including PC-3. Their findings also confirmed the selective induction of apoptosis, with minimal necrosis, which aligns with our results showing that holothurin B saponin induces early apoptosis in PC-3 and PANC-1 cells, avoiding necrosis. This study contributes to the increasing amount of data demonstrating the cytotoxic potential of saponins from sea cucumbers with their capacity to specifically activate apoptotic pathways without inducing necrotic cell death (Pranweerapaiboon et al., 2021). While Wong et al. (2016) previously found that saponins from *Holothuria leucospilota* had similar apoptotic effects in cancer cells, our research offers a more comprehensive understanding of early apoptosis processes and their dose-dependent features. Although Nguyen et al. (2018) showed that sea cucumber saponins may trigger apoptosis through the MAPK signaling pathway, their findings included both necrotic and apoptotic effects, suggesting a more comprehensive manner of cell death. However, our investigation revealed that holothurin B saponin exclusively activates this route, focusing on its early apoptotic effects. This implies a more controlled and targeted therapeutic impact, which could reduce negative consequences such inflammation or necrosis-related tissue damage (Nguyen et al., 2018). Research by

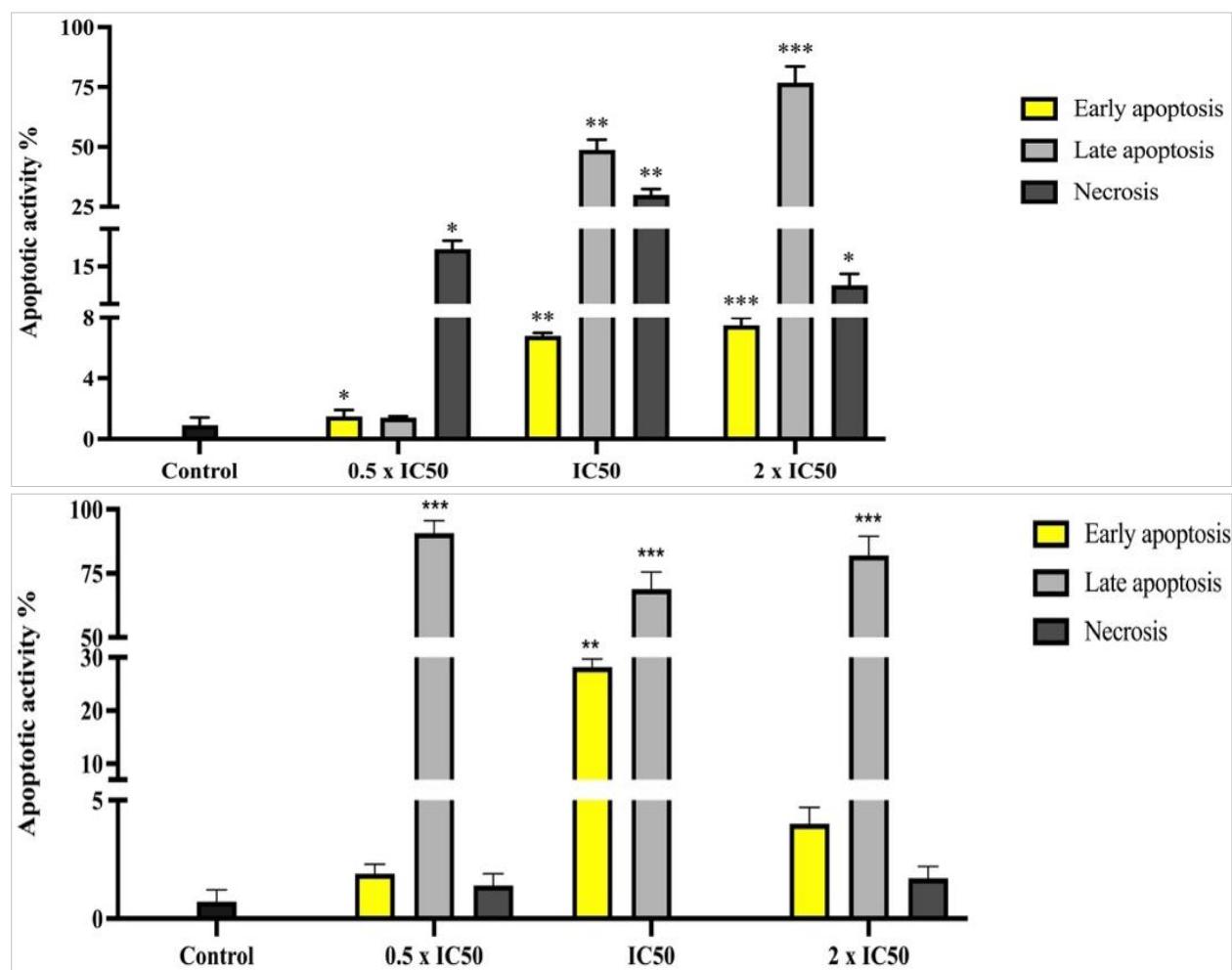


Figure 3. Apoptotic activity of holothurin B saponin on a) PC-3 and b) PANC-1 cells after 48 h of treatment (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Cui et al. (2020), who examined saponins from *H. scabra* and verified their anti-proliferative and pro-apoptotic effects on a variety of cancer cell lines, including HepG2 and MCF-7, provides more evidence. But, their research did not thoroughly examine the underlying apoptotic pathways. Our research, on the other hand, focused on the particular early-stage apoptotic events and their dose-related dynamics, providing a more in-depth comprehension of these cellular mechanisms. While Li et al. (2021) studied saponins from *H. mexicana* in colon cancer models and found both apoptotic and necrotic effects, our results are notable for indicating a more selective and focused apoptotic response. Furthermore, Liu et al. (2022) evaluated the anticancer potential of saponins extracted from *H. nobilis*, demonstrating a notable suppression of cell growth in lines like A549 and HeLa. They also discovered that the mitochondrial pathway is the mechanism *via* which apoptosis is induced. While this aligns with the apoptotic effects observed in our study, holothurin B saponin's exclusive induction of early apoptosis, with lower involvement of the mitochondrial pathway or necrosis, provides a unique advantage, suggesting that it may be more specific and less harmful to normal tissues (Liu et al. 2022).

Holothurin B saponin induced premature apoptosis in PANC-1 cells to a maximum, thus diminishing the risk of necrosis-induced inflammation and tissue damage. This characteristic indicates that holothurin B saponin may serve as an excellent candidate for future *in vivo* and clinical studies because it could lead to fewer side effects compared to agents that induce necrosis.

Conclusions

Holothurins are the important components in sea cucumber species *H. tubulosa* which is common in the Aegean Sea. The amount of holothurin B (5) was calculated to be a significant compound among the saponins isolated from the dry material with a yield of 0.0211%. Considering the obtained yield, along with the pharmaceutical potential of sea cucumbers and the overall export unit value, the importance of this study becomes evident in highlighting the economic and biomedical relevance of *H. tubulosa*-derived holothurins. Although sea cucumber fishery in Türkiye was made since 1996, there are no application on the pharmaceutical usage of the isolated compounds and/or extracts from these echinoderms. In this study, we first evaluated the cytotoxicity of holothurin B on normal cells (CCD-34Lu) and observed no significant toxic effect at concentrations up to 50 μ M. Based on these findings, we proceeded to test holothurin B on various cancer cell lines, where it showed promising cytotoxic activity. For holothurin B, which was isolated in significant amounts from *H. tubulosa* in this study, further *in vivo* and mechanistic studies can pave the way for these pharmaceutical applications. However, these additional

investigations are necessary to fully understand holothurin B's mode of action and therapeutic applicability. This provides a deeper insight into the apoptotic mechanisms of holothurin B saponin, making it a key contribution to the understanding of sea cucumber saponins in cancer research.

Ethical Statement

This study meets the ethical guidelines outlined by the American Fisheries Society.

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Author Contribution

Gulce Gucur: Investigations; Writing original draft; Formal analysis. Sevda Zeinali: Investigations; Writing original draft. Deniz Gunay: Resources. Ayse Nalbantsoy: Methodology, Writing – Review & Editing; Formal analysis. Nazli Boke Sarikahya: Conceptualization; Methodology, Formal analysis, Writing – Review & Editing; Supervision.

Conflict of Interest

The authors declared that there is no conflict of interest.

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