

Evaluation of cytotoxic, apoptotic effects and phenolic compounds of sea cucumber *Holothuria tubulosa* (Gmelin, 1791) extracts

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Abstract: This study aimed to investigate the potential cytotoxic effects and phenolic contents of aqueous (HTS) and methanolic (HTM) extracts of *Holothuria tubulosa* gathered from Muğla (Turkey) against different cancer cells for the first time. An MTT assay was carried out to determine cellular viability. Apoptotic cell death was determined using flow cytometry. DNA fragmentation assay and caspase-3 activity analysis were carried out. Both extracts were analyzed by high performance liquid chromatography (HPLC) for quantification of phenolic compounds. Both HTS and HTM extracts generally caused a decrease in the cell viability in a concentration- and time-dependent manner. The lowest IC₅₀ value was found in the HeLa cell line with 21.01 µg/mL for the HTS extract at 72 h. Both extracts are capable of significantly enhancing the apoptotic process in HeLa and A549 cells, causing DNA fragmentation, and also increasing activity of caspase-3 in both these cell lines, especially after treatment with at 500 µg/mL. Based on HPLC analysis, the most abundant component was epicatechin (790.091 µg/g extract) and 2.5 dihydroxybenzoic acid (153.890 µg/g extract) in the HTS and HTM extracts, respectively. This is the first study showing potential cytotoxic activity of *H. tubulosa* extracts from Muğla, Turkey against human cancer cells. Our results suggest that *H. tubulosa* may be a good source for development of anticancer agents.

Key word: *Holothuria tubulosa*, cancer cell lines, cytotoxicity, apoptosis, HPLC analysis

1. Introduction

Cancer remains one of the most significant causes of death worldwide, resulting in the morbidity and mortality of millions of people [1]. Most currently available drugs have the potential to display toxic side effects without cell selectivity for cancer cells [2]. Moreover, resistance to chemotherapeutic drugs may develop in cancer cells [3]. Therefore, studies for determination of new anticancer agents that can selectively kill malignant tumor cells remain important [4]. Additionally, an optimal anticancer agent is also expected to delay or reverse cancer development [5].

Phenolic compounds, widespread in plant-based foods, are known to function as antioxidants. They are capable of contributing to the scavenging of free superoxide radicals, reducing the risk of cancer, and also protecting vital biomolecules such as proteins, lipids, and DNA from the damaging effects of oxidative processes [6].

Most (~60%) approved drugs for cancer treatment originate from natural products. Marine organisms make up about half of the total species diversity on the earth. The marine ecosystem is a significant source for discovery of useful therapeutic agents [7], and marine environments contain numerous species possessing a wide range of novel

bioactive secondary metabolites [8]. Natural compounds from marine sources may exhibit various bioactive properties such as antitumor, anticancer, antimicrotubule, antiproliferative, antihypertensive, and cytotoxic effects [9]. Currently, roughly 28,600 natural products have been reported to originate from various marine sources [10].

Sea cucumbers (or holothurians) are classified as echinoderms (*phylum Echinodermata*) and belong to the class Holothuroidea. They are a group of marine invertebrates found in benthic areas and deep seas that usually have leathery skin and an elongated body which is soft and looks like a cucumber. Sea cucumbers, also known as trepang, bêche-de-mer, balate or haishen (ginseng of the sea), are used as food and in Asian folk medicine [11]. The various biological and pharmacological benefits of sea cucumbers such as their anticoagulant, antithrombotic, antioxidant, anticancer, and antimicrobial properties were reviewed by Bordbar et al. [12] and Khotimchenko [13]. Because sea cucumbers have nutritional, health and possibly therapeutic value, they have attracted the attention of scientists in recent decades [13]. The compounds responsible for the biological activities of sea cucumbers have been reported to be chondroitin sulfate,

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glycosaminoglycans, peptides, and other secondary metabolites, as well as triterpene glycosides called saponin [12,14].

Sea cucumbers have been exported from Turkey since 1996. One main commercial species in Turkey is *Holothuria tubulosa* (Gmelin, 1791) [15]. Biodiversity is largely threatened by global warming and other environmental causes. As a result, it is important to investigate local natural sources in detail for possible use in the development of drugs [16]. To our knowledge, there is a lack of information about the potential anticancer properties of *H. tubulosa* in the literature, although there are different studies related to its population, reproduction, and biometric traits [15,17]; cytotoxic activity [18], antiinflammatory activity [19], antioxidant, and antimicrobial activity [20]; immune mediators [21], proximate composition, and fatty acid profile [22]. Therefore, the aim of this study is to evaluate the potential cytotoxic and apoptotic effects of aqueous (HTS) and methanolic (HTM) extracts of *H. tubulosa* from Muğla, Turkey on different human cancer cell lines, as well as determining the phenolic compounds in the extracts by high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Collection of and extract preparation from *H. tubulosa*

H. tubulosa specimens were collected from the Gökova Gulf in Muğla, Turkey and transported to the laboratory. The internal organs of the sea cucumbers were immediately removed. After washing their remaining parts with tap water and then deionized water, the sea cucumbers' bodies were stored at -80°C or extracted. For extraction, the sea cucumber samples were sliced, dried, and minced into small pieces. Approximately 10 g of each minced sample was extracted with 100 mL of deionized water or methanol (Merck, USA) and shaken at 30°C for 8 h at least 3 times and then filtered. The methanol used as a solvent was removed by rotary evaporation (IKA, RV 10, USA) at 40°C . Finally, after the lyophilization process, each crude extract (HTS or HTM extract) that was obtained was kept at -80°C in the dark. The stock solution of each crude extract was prepared by suspending it in growth medium containing 10% DMSO.

2.2. Cell cultures for human cancer and normal cell lines

All cell lines used in this study were obtained from ATCC. These cells were A549 (lung adenocarcinoma), HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma), PC-3 (prostate adenocarcinoma) human cancer cell lines, HEK293 (normal human embryonic kidney), and BEAS-2B (normal bronchial epithelium) cell lines. The cell lines were cultured in an RPMI-1640 medium with stable L-glutamine (Biochrom, Germany), 10% heat inactivated fetal bovine serum (FBS) (Biochrom, Germany), 100

units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin sulphate (Biochrom, Germany) and incubated at 37°C under a humidified atmosphere of 5% CO_2 .

2.3. MTT assay

The effects of the extracts on cell viability were assessed based on an MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide) assay [23]. The cell lines were seeded at a concentration of 4×10^3 cells/well in 96-well plates and allowed to attach for 24 h. The cells used were then treated with the HTS or HTM extract of *H. tubulosa* at 8 different concentrations (1000–7.8 $\mu\text{g}/\text{mL}$) for 24, 48, and 72 h. The untreated cells were used as a control group. At the end of the treatment periods, the medium with the extract in each well was replaced with 100 mL of fresh growth medium. The cells were later incubated with 10 μL of 5 mg/mL MTT reagent (Applichem, USA) in phosphate-buffered saline (PBS) for 4 h. After removing the contents of each well, 100 mL of DMSO was added to dissolve the purple crystals of formazan formed in the viable cells. Finally, absorbance (Abs) was measured by a microplate reader (Thermo Scientific, Multiskan FC, USA) at 540 nm. Because the serial dilutions were prepared in growth medium from stock solutions, the final concentration of DMSO in the tested cells was no more than 0.1%. Each MTT assay was performed in triplicates at least 2 times. The percent of the cell viability was calculated according to the following formula:

$$\text{Cell viability \%} = (\text{Mean Abs of treated cells} / \text{Mean Abs of control or untreated cells}) \times 100.$$

In this study, the degree of selectivity of the cytotoxic extracts was expressed as selectivity index (SI) = IC_{50} in normal cells/ IC_{50} in cancer cells, according to the information reported in a previous study [24].

2.4. Analysis of apoptotic cells

The percentage of apoptosis in the treated (A549 and HeLa) and control cells were determined by Annexin V-FITC/propidium iodide staining using flow cytometry, according to the instructions of the Annexin V-FITC Apoptosis Detection Kit (eBioscience, USA) protocol. The cells with a density of 5×10^5 cells/well were cultured in 6-well plates and incubated for 24 h. After treatment with the HTS or HTM extract at various concentrations (500, 250, and 125 $\mu\text{g}/\text{mL}$) for 24 and 48 h (and 72 h for A549 cells only), cell suspensions were harvested and washed twice with cold PBS. The cells were then resuspended in 190 μL binding buffer and incubated with 5 μL Annexin V-FITC at room temperature for 10 min in the dark. After centrifugation, the cells were again dissolved in 190 μL binding buffer and stained with 10 μL propidium iodide at 20 $\mu\text{g}/\text{mL}$ at room temperature in the dark. Finally, the cells in each group were evaluated by BD FACSCanto flow cytometry (BD Biosciences, San Jose, CA, USA) using

the BD FACSDiva software v6.13. The control cells were untreated cells.

2.5. DNA fragmentation assay

DNA extraction was performed based on the procedure described by Gong et al. [25] with modification. The A549 and HeLa cell lines were cultured in 6 mL of growth medium in 25 cm² cell culture flasks at a concentration of 7.5×10^4 cells for 24 h. The cells were then treated with the HTS or HTM extract at 500, 250, and 125 µg/mL concentrations for 36 h. The untreated cells were used as a control group. After this, the cells were harvested by scraping and washed with DPBS. The cells were later prefixed in 70% ethanol at -20 °C for 24 to 72 h. After centrifugation, the ethanol was thoroughly removed, and each pellet was extracted with 50 µL of phosphate-citrate buffer at pH 7.8 and 37 °C for 30 min. After centrifugation, the supernatant, at a volume of about 40 µL, was collected and incubated with 5 µL RNase (20 mg/mL) and 5 µL Tween 20 at 37 °C for 30 min. Following this, proteinase K (20 mg/mL) was added and incubated additionally for 30 min at 37 °C. For DNA fragmentation analysis, the DNA was subjected to electrophoresis at 80–100 V in 1.5% agarose gel stained with ethidium bromide and finally visualized by a UV transilluminator (Vilber Lourmat, France).

2.6. Caspase-3 activity assay

Caspase-3 activity was detected using a colorimetric assay kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. The A549 and HeLa cells were plated at 2×10^6 cells/well in 6-well plates. After 24 h of incubation, the cells were treated with the HTS or HTM extract at various concentrations (500, 250, and 125 µg/mL) for 36 h. At the end of the incubation time, the cells were collected, resuspended in 50 µL of cold cell lysis buffer, and incubated on ice for 10 min. After centrifuging at 10,000 g for 1 min, the protein concentration of each cell lysate was determined by Bradford's method [26]. Approximately 50 µL of 2X reaction buffer containing 10 mM DTT and 5 µL of caspase-3 substrate (4 mM DEVD-*p*-NA) was added to each sample containing 200 µg of protein and incubated at 37 °C for 2 h. The absorbance of *p*-NA was measured at 405 nm in a microplate reader. The caspase-3 activity of untreated cells was determined as 1.0, and data related to caspase-3 activity was expressed as a fold-increase by comparing them to that of the untreated cells.

2.7. Analysis of phenolic compounds by HPLC

Phenolic compounds of the HTS and HTM extracts were analyzed by HPLC (Shimadzu Scientific Instruments, Kyoto, Japan) according to method described by Caponio et al. [27] with a slight modification. The analysis was performed with a photodiode array detector (SPD-M20A), LC20 AT pump, and a SIL-20ACHT auto sampler. The separation was carried out using the C18-column

with a CTO-10ASVp column oven. The mobile phases were solvent A: 3.0% formic acid in distilled water and solvent B: methanol. Each sample of 0.2 g was dissolved in a mobile phase and filtered through a 0.45-µm filter before injection into the HPLC. Gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 2,5 dihydroxybenzoic acid, chlorogenic acid, vanillic acid, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, cinnamic acid, and quercetin were used as standards. Differentiation and quantitative analysis were based on comparison of the standards. The amount of each phenolic compound was expressed as µg/g per gram of the extract.

2.8. Statistical analysis

All calculation of the IC₅₀ values were carried out using the GraphPad Prism software version 7.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. The cytotoxic effects of *H. tubulosa* extracts on different cell lines

An MTT assay was used to assess the potential cytotoxic effects of the extracts against human cancer (A549, HeLa, PC-3 and MCF-7) and normal cell lines (HEK293 and BEAS-2B). According to our results, it may be suggested that the HTS and HTM extracts caused a concentration- and time-dependent decrease in cell viability in comparison to the control groups (Figure 1). Cell proliferation of the tested cancer cells was markedly inhibited by the HTS and HTM extracts between concentrations of 250 and 1000 µg/mL after 24, 48, and 72 h of treatment. The approximate 50% inhibitory concentration (IC₅₀) of both extracts for all cells that were used and SI values are summarized in Table 1 and Table 2, respectively. The lowest IC₅₀ values among all cancer cells at 72 h were 21.01 µg/mL in the HeLa cells for the HTS extract and 63.12 µg/mL in the A549 cells for the HTM extract.

Based on the IC₅₀ values, it may be stated that the HTS extract showed a higher cytotoxic effect against all cancer cell lines tested at 24 and 48 h in comparison to normal cell lines. Likewise, the HTM extract at 24 and 48 h had a higher cytotoxic effect against the A549 and HeLa cancer cells than the other cancer and normal cell lines. Furthermore, SI values were calculated by comparing the IC₅₀ values of the extracts in the normal cell lines to the IC₅₀ values of the same extracts in the cancer cell lines. As Nguyen and Ho-Huynh [24] stated, an SI value above 2 is considered cytotoxic selectivity. As shown in Table 2, the HTS extract showed the best SI values against the A549 cell line (4.12 and 3.48, respectively) for 48 h versus both the HEK293 and the BEAS-2B cell lines and against the HeLa cell line (2.85) for 72 h versus the BEAS-2B cell lines. Additionally, the best SI value determined for the HTM

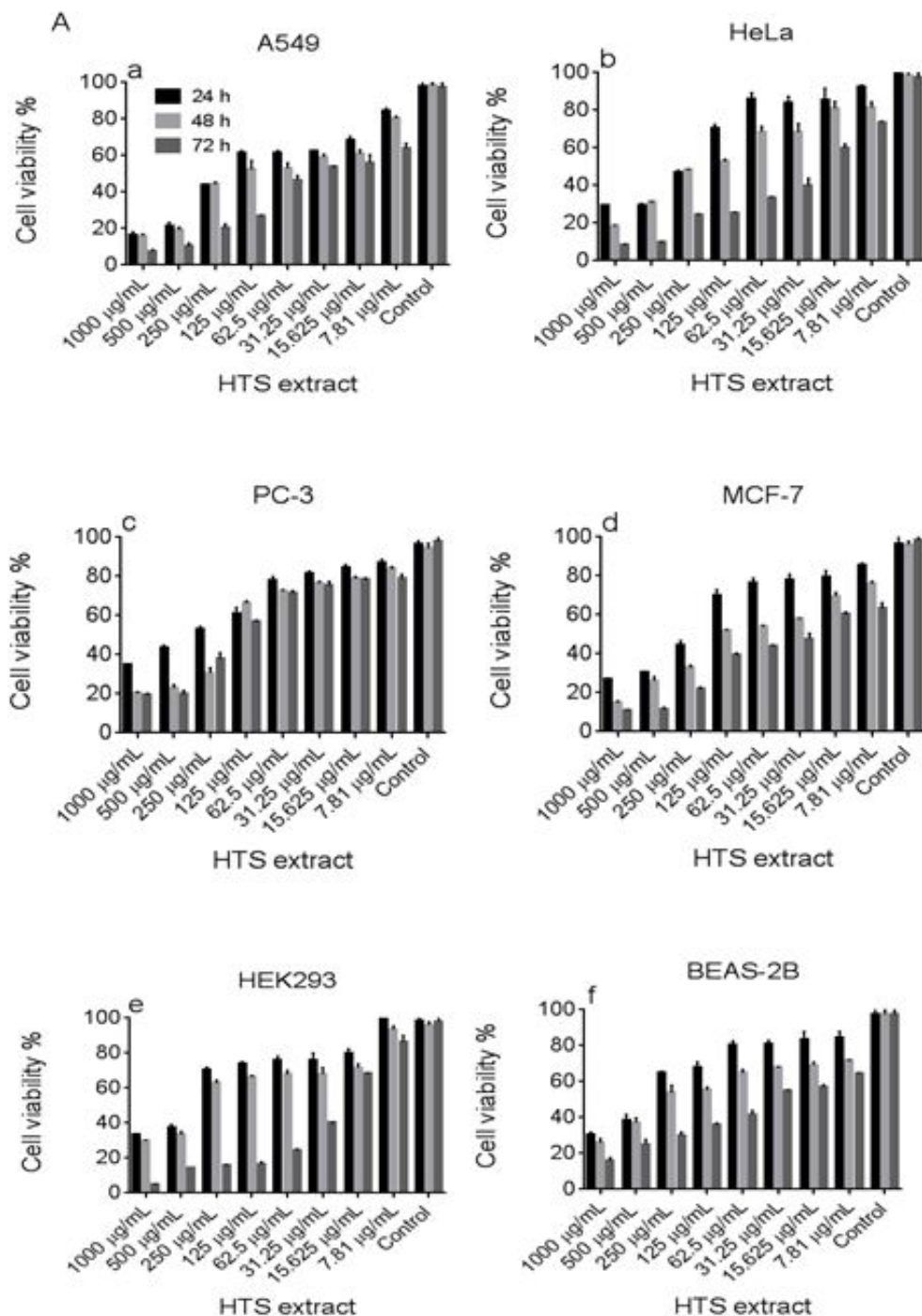


Figure 1. Cell viability of the cells after exposure to HTS (A) and HTM (B) extracts on different human cancer and normal cells. A549 (a), HeLa (b), PC-3 (c), MCF-7 (d), HEK293 (e), BEAS-2B (f) cells were treated with the HTS or HTM extract in a concentration and time dependent manner. Cell viability was evaluated by MTT assay. Data was expressed as mean \pm standard error of 3 separate experiments.

extract was against the HeLa (2.61) for 48 h versus the HEK293 cell line. Altogether, these results demonstrated that both extracts may be tested to obtain promising anticancer agents.

3.2. The effects of *H. tubulosa* extracts on apoptosis

The HTS and HTM extracts exhibited more cytotoxicity on the A549 and HeLa cells in general. As a result, the effects of both extracts on apoptosis were investigated by

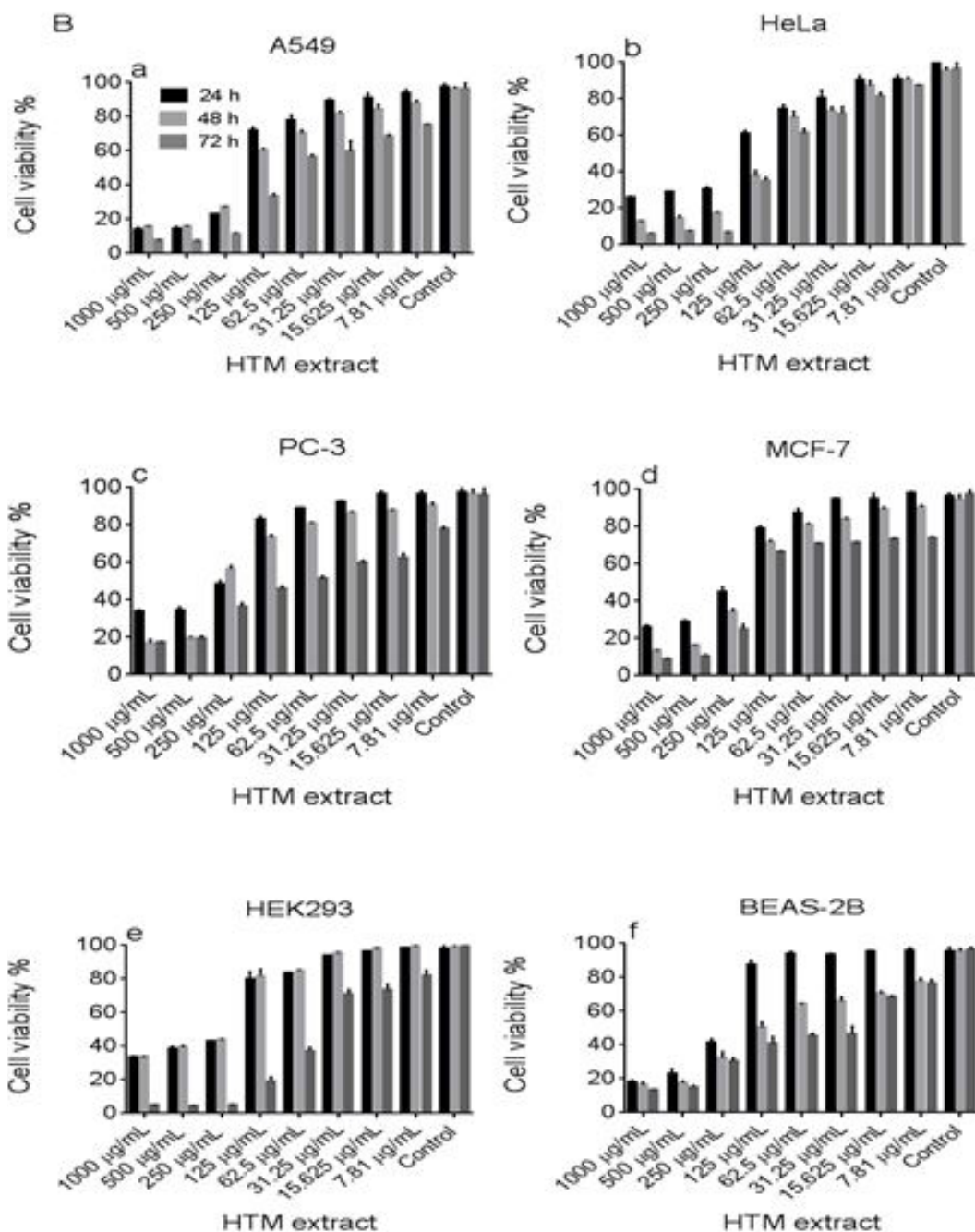


Figure 1. (Continued).

flow cytometry using AnnexinV/PI double staining in these 2 cell lines. As shown in Figure 2A(a), treatment of A549 cells with 500, 250, and 125 µg/mL of the HTS extract for 48 and 72 h resulted in a significant increase in the percentages of apoptosis (quadrants 2 and 4) and reduced the percentages of the viable cells (quadrant 3) in comparison to the control. The percentages of apoptotic A549 cells at 48 h were increased from 3.6% (control) to 42.2%, 36.8%, and 35.7% for 500, 250, and 125 µg/mL

concentrations of the HTS extract, respectively. After treatment with 500, 250, and 125 µg/mL concentrations of the HTS extract for 72 h, the percentages of the apoptotic A549 cells were 45.2%, 48.7%, and 47.7%, respectively, in comparison to the control group (8%). As shown in Figure 2A(b), treatment with 500 µg/mL of the HTM extract altered the percentages of the apoptotic A549 cells from 20% (control) to 56.1% for 24 h and from 8% (control) to 47.4% for 72 h. Furthermore, the HTM extract at 500 µg/

Table 1. Cytotoxic effects of (approximate IC₅₀ value) *H. tubulosa* extracts on A549, HeLa, PC-3, MCF-7, HEK293, and BEAS-2B human cell lines.

Cells	IC ₅₀ (µg/mL)					
	HTS extract			HTM extract		
	24 h	48 h	72 h	24 h	48 h	72 h
A549	229.5	112.8	53.95	200.3	157.1	63.12
HeLa	238.3	180.2	21.01	146.4	83.73	83.32
PC-3	282.6	177.2	166.6	282.3	257.2	70.03
MCF-7	228.5	176.7	36.15	267.6	221.5	179.6
HEK293	479	464.4	28.11	226.4	218.2	59.47
BEAS-2B	413.5	393.05	59.80	241.15	168.9	68.19

Table 2. Selectivity index (SI) values of the *H. tubulosa* extracts.

Cells	SI*	HTS extract			HTM extract		
		24 h	48 h	72 h	24 h	48 h	72 h
		A549	vs HEK293	2.08	4.12	0.52	1.13
	vs BEAS-2B	1.8	3.48	1.10	1.20	1.07	1.08
HeLa	vs HEK293	2.01	2.57	1.33	1.54	2.61	0.72
	vs BEAS-2B	1.73	2.18	2.85	1.64	2.01	0.81
PC-3	vs HEK293	1.69	2.62	0.16	0.80	0.84	0.84
	vs BEAS-2B	1.46	2.21	0.35	0.85	0.65	0.97
MCF-7	vs HEK293	2.09	2.62	0.77	0.84	0.98	0.33
	vs BEAS-2B	1.8	2.22	1.65	0.90	0.76	0.37

*SI values determined for A549, HeLa, PC-3, and MCF-7 cancer cells versus normal HEK293 and BEAS-2B cell lines. The numbers in bold were expressed as important SI values.

mL caused the most significant increase in the apoptotic A549 cells (90%) in comparison to control (3.6%) at 48 h. However, the HTM extract at concentrations of 250 and 125 µg/mL resulted in less apoptosis in the A549 cells than the HTS extract at the same concentration for 48 and 72 h. Additionally, our results showed that the apoptotic cells in the treated A549 cells for 48 h belonged predominantly to those in a late stage of apoptosis.

Since the percentages of the necrotic cells were generally increased after 72 h of treatment, the flow cytometry analysis of the HeLa cells was carried out for only 24 and 48 h. The HTS extract generally caused increased apoptotic effects against the HeLa cells in a time-dependent manner as in the A549 cells (Figure 2B). After exposure to 500 µg/mL of the HTS extract, the apoptosis rate in the HeLa cells increased from

10.5% in the control to 25.9% for 24 h and from 7.3% (control) to 44.3% for 48 h. Additionally, the percentages of the apoptotic HeLa cells treated with 250 µg/mL of the HTS extract were 19.7% and 50.2% for 24 and 48 h, respectively. The HTS extract at 125 µg/mL also induced a higher apoptosis rate in the HeLa cells at 48 h (44.3%) than at 24 h. As shown in Figure 2B(b), the HTM extract induced apoptosis in 31.6% and 49.6% of the HeLa cells exposed to 500 µg/mL for 24 and 48 h, respectively. The HTM extract at concentrations of 250 and 125 µg/mL did not cause a notable increase in the percentage of apoptotic HeLa cells for 24 and 48 h in comparison to the control groups. As observed in the A549 cells, the apoptotic cells among the HeLa cells treated for 48 h were found to be in the late stage of apoptosis. These findings may show that *H. tubulosa* has cytotoxic properties.

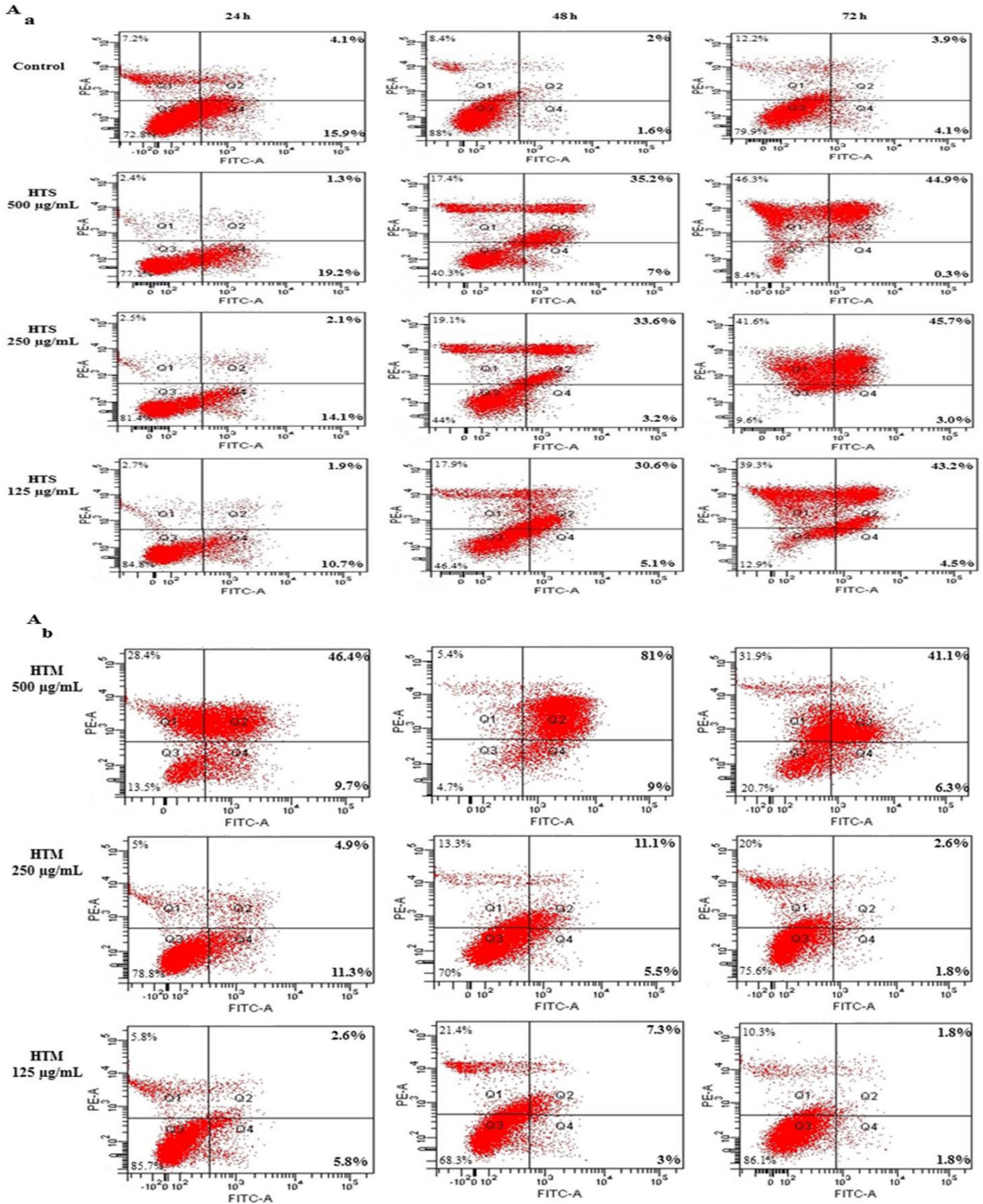


Figure 2. Flow cytometry analysis of apoptosis in A549 (A) and HeLa (B) cells treated with HTS (a) or HTM (b) extract. Cells were separately treated with each extract at 500, 250, and 125 µg/mL at different incubation times. Annexin V/PI double-staining assay was carried out and cells were analyzed by flow cytometry. Untreated cells served as control. Cells were distributed into 4 quadrants: viable cells (Q3), early apoptotic cells (Q4), late apoptotic cells (Q2), and necrotic cells (Q1).

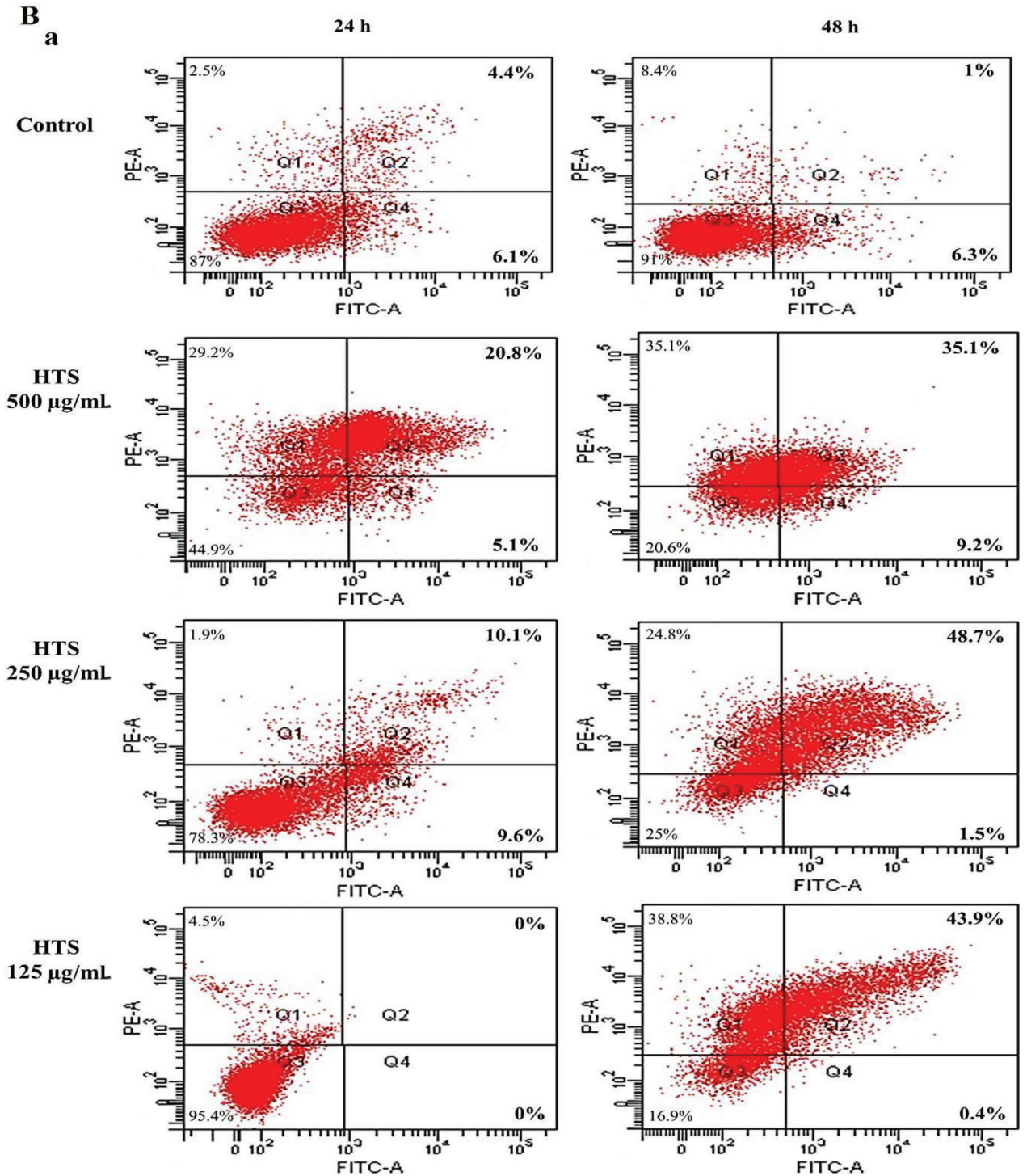


Figure 2. (Continued).

3.3. Determination of DNA fragmentation in treated cells with *H. tubulosa* extracts

DNA fragmentation assay was also carried out to confirm the induction of apoptosis arising from the HTS and HTM extracts. After treatment for 36 h, DNA fragmentation

known as the characteristic of apoptosis [28] was observed on the A549 and HeLa cells. The prominent DNA ladder was found in the A549 cells treated with 250 µg/mL of the HTS or HTM extract (Figure 3). Therefore, these results confirmed that the HTS and HTM extracts may induce apoptosis.

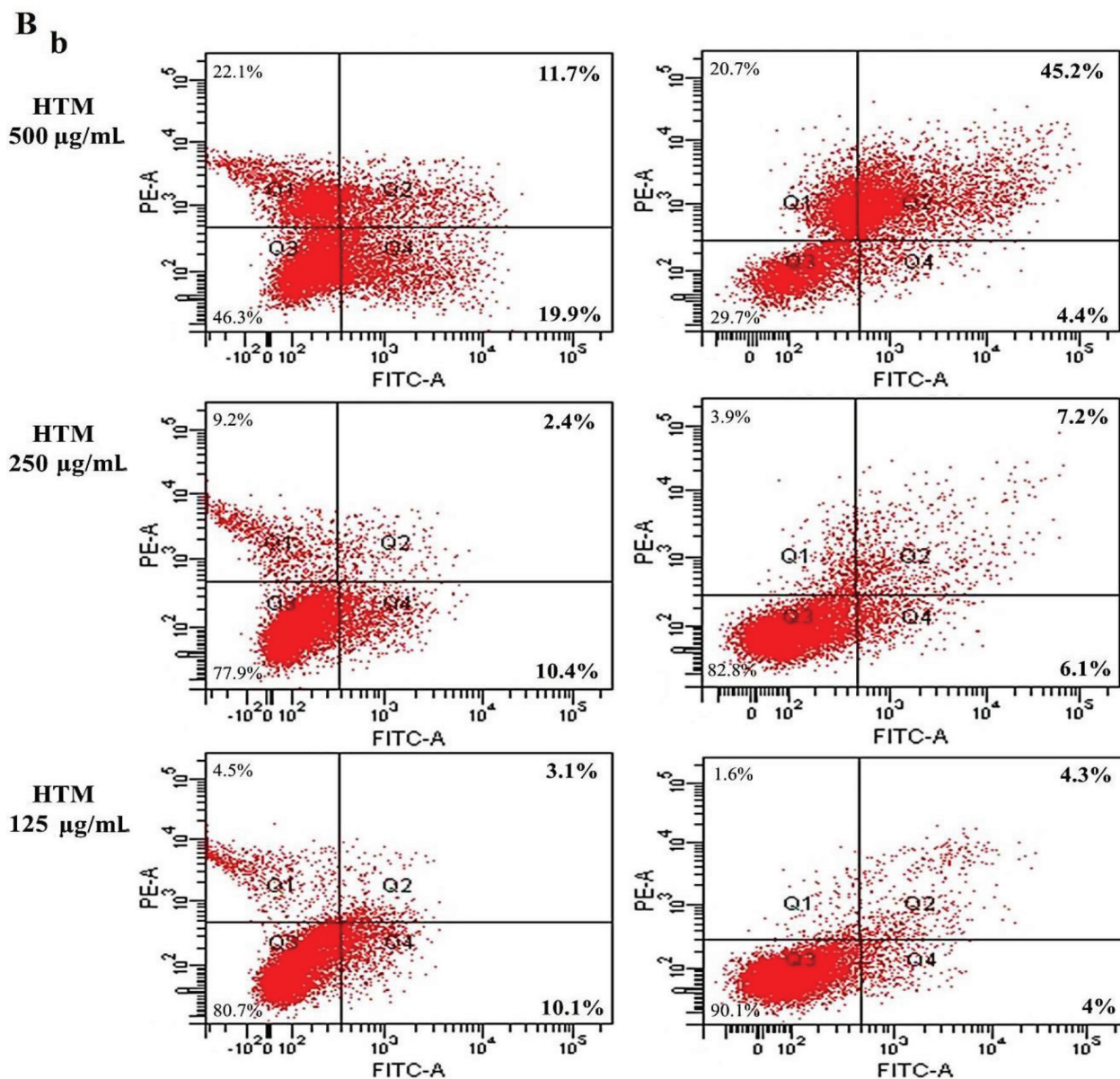


Figure 2. (Continued).

3.4. The effects of *H. tubulosa* extracts on caspase-3 activity

Caspase-3 activities in the treated and untreated cells were analyzed using an in vitro colorimetric substrate DEVD-pNA. The results shown in Figure 4A reveal that the HTS extract at 500 µg/mL caused about 6.7-fold and 7-fold increases in the caspase-3 activity in the A549 and HeLa cells, respectively, in comparison to the untreated cells. Moreover, after being treated with 500 µg/mL of the HTM extract (Figure 4B), the caspase-3 activity in the A549 and HeLa cells was respectively about 7.6-fold and 6-fold higher than those in the untreated cells. Another significant caspase-3 activity, which was approximately

a 2.8-fold increase in comparison to the untreated cells, was detected in the HeLa cells exposed to the 250-µg/mL concentration of the HTS extract. These data help to confirm the results obtained from the analysis of flow cytometry and DNA fragmentation.

3.5. Phenolic composition in *H. tubulosa* extracts

The phenolic compounds and quantities were characterized by HPLC using 15 standards from the HTS and HTM extracts, as shown in Table 3. According to this analysis, 13 and 12 phenolic compounds were determined in the HTS and HTM extracts, respectively. The main phenolic compounds in the HTS extract were detected as epicatechin (790.091 µg/g extract), ellagic acid (558.671

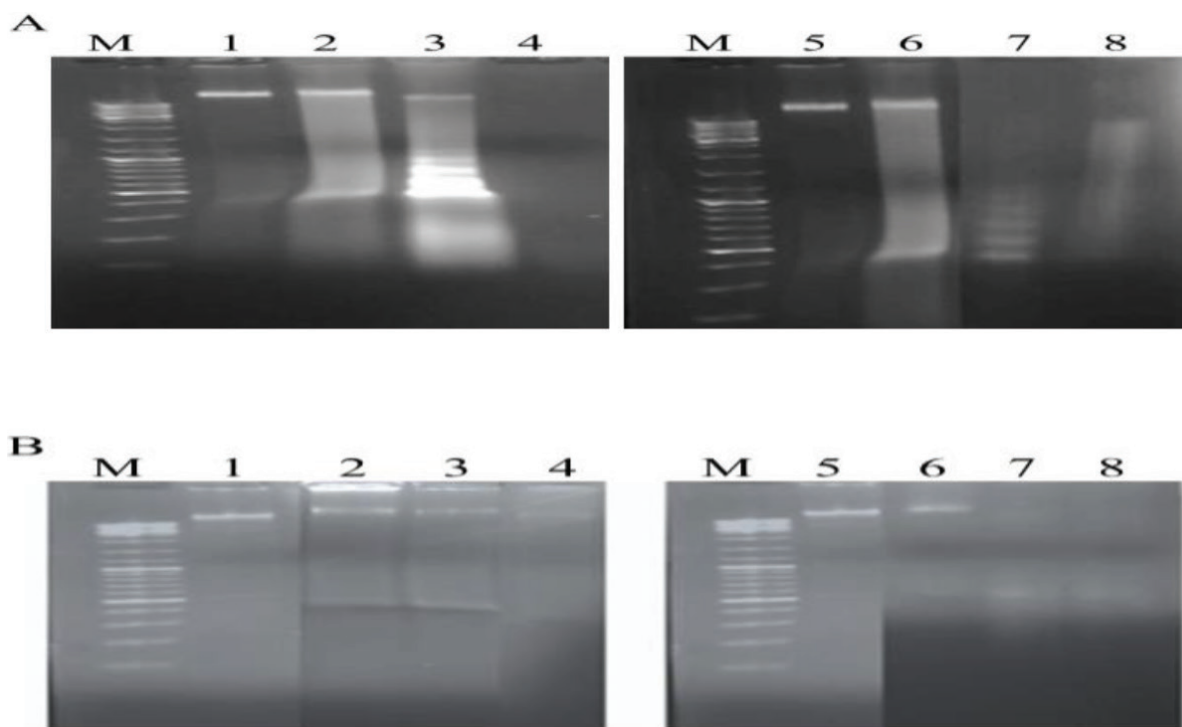


Figure 3. DNA fragmentation of A549 (A) and HeLa (B) cells exposed to *H. tubulosa* extracts. The cells were treated with increasing concentrations of HTM (2–4) or HTS (6–8) extracts of 125, 250, and 500 µg/mL, respectively. M: Marker 1kb DNA Ladder, 1 and 5: control (untreated cells).

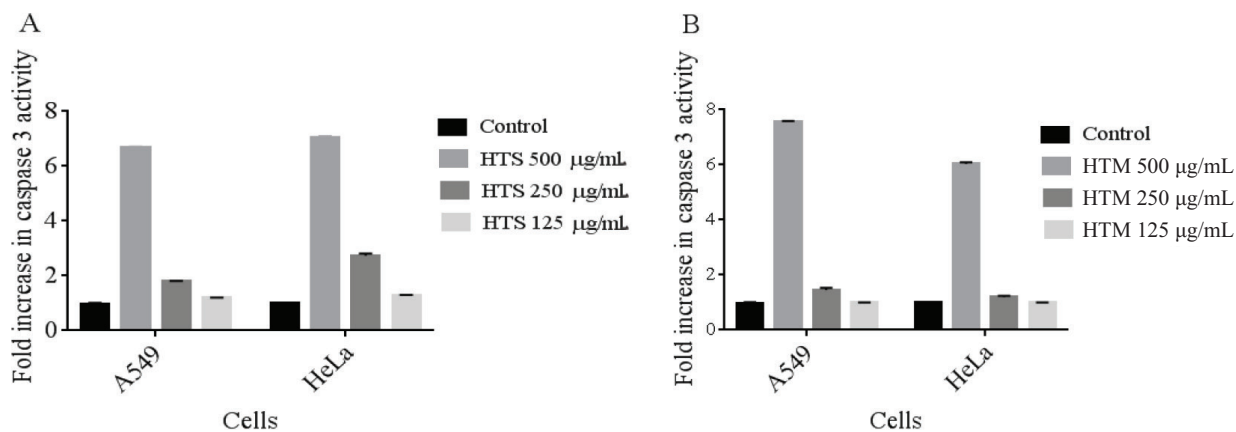


Figure 4. Measurement of caspase-3 activity in HTS (A) or HTM (B) treated A549 and HeLa cells. The caspase-3 activities in control (untreated) cells are taken as 1-fold and other activities were presented by comparing with the control cells. Data was expressed as mean \pm standard error of 3 separate experiments.

µg/g extract), gallic acid (205.871 µg/g extract), and 2,5 dihydroxybenzoic acid (130.541 µg/g extract)—all listed based on their amounts. The HTM extract was also found to be rich in 2,5 dihydroxybenzoic acid (153.890 µg/g extract), gallic acid (133.169 µg/g extract), and ellagic acid (109.258 µg/g extract), except for epicatechin (0.726 µg/g extract). *p*-coumaric acid was found in the HTS extract (78.475 µg/g extract), but it was not seen in the HTM

extract. Cinnamic acid was assessed as a minor compound for both extracts. Neither rutin nor quercetin was found in the extracts.

4. Discussion

Sea cucumbers are reportedly used as foods and as a traditional remedy for treating various diseases in various countries such as Korea, Japan, China, Malaysia, and

Table 3. HPLC analysis of HTS and HTM extracts for phenolic compounds.

No.	Phenolic Compounds	RT (min)	UV _{max} (nm)	HTS (µg/g extract)	HTM (µg/g extract)
1	Gallic acid	6.8	280	205.871	133.169
2	3,4-dihydroxybenzoic acid	10.7	280	28.264	4.699
3	4-hydroxybenzoic acid	15.7	280	11.171	15.464
4	2,5-dihydroxybenzoic acid	17.2	320	130.541	158.890
5	Chlorogenic acid	18.2	320	5.638	11.91
6	Vanillic acid	19.2	320	3.423	7.483
7	Epicatechin	21.3	260	790.091	0.726
8	Caffeic acid	22.7	280	8.369	8.594
9	<i>p</i> -coumaric acid	26.1	320	78.475	-
10	Ferulic acid	30.1	320	0.391	0.208
11	Rutin	45.6	360	-	-
12	Ellagic acid	47.7	240	558.671	109.258
13	Naringin	49.7	280	0.387	0.379
14	Cinnamic acid	67.8	280	0.013	0.013
15	Quercetin	71.1	360	-	-

RT: retention time.

Indonesia [29]. Because of the bioactive compounds they carry, they continue to be considered as potential sources of novel bioactive agents for the prevention and treatment of various diseases [30–32]. There are several studies on the cytotoxic effects of different sea cucumber species on various cancer cell lines in the literature [33–36].

Our literature review shows that there is no previous research-based evidence about the biological activities related to the anticancer properties of 2 different extracts of *H. tubulosa* growing in Turkey: HTS and HTM. Their cytotoxic effects on 4 cancer cell lines (A549, HeLa, MCF-7, and PC-3) and 2 normal cell lines (HEK293 and BEAS-2B) were conducted in MTT assays at varying concentrations and different time intervals. Following the comparison of the IC₅₀ values of both extracts at 24 and 48 h, it was observed that these are lower for HTS in all cancer cell lines and for HTM in only A549 and HeLa cells than in the 2 normal ones. The lowest IC₅₀ values of the HTS were calculated as 112.8 µg/mL for the A549 at 48 h and 21.01 µg/mL for the HeLa at 72 h. The highest cytotoxic effect for HTM extract was determined in the HeLa cells with IC₅₀ value of 83.73 µg/mL at 48 h. SI values above 2 refer to the cytotoxic selectivity [24]; the highest SI values for the HTS were detected in A549 cells at 48 h and HeLa cells at 72 h and those for the HTM in the HeLa at 48 h. Unlike our study, Luparello et al. [18] showed that the total and filtered HTS extracts of the coelomic fluid of *Holothuria tubulosa* gathered from the Gulf of Palermo (Sicily, Italy)

are cytotoxic to TNBC (triple negative breast cancers) cell line MDA-MB231. The authors also reported that their study provided the first data linking cell exposure to *H. tubulosa*-derived extracts to the event of autophagy induction. In addition, there are various studies in the literature on the cytotoxic effects of different *Holothuria* species on cancer cells. Althunibat et al. [35] assessed the inhibition effect of different sea cucumber extracts (*Holothuria scabra*, *Holothuria leucospilota*, and *Stichopus chloronotus*) on the growth of 2 cancer cell lines (A549 and C33A) and reported that the aqueous extract of *S. chloronotus* was more toxic against C33A cells (IC₅₀ = 10.0 µg/mL) than A549 (IC₅₀ = 28.0 µg/mL). Among organic extracts, *H. scabra* was found to be the one with the highest antiproliferative effects against both A549 (IC₅₀ = 15.5 µg/mL) and C33A (IC₅₀ = 3.0 µg/mL) cells. Furthermore, 3 triterpene glycosides (scabraside D, fuscocineroside C, and 24-dehydroechinoside A) from *H. scabra* showed a significant cytotoxic effect on P-388 (a mouse leukemic cell), A549, MKN-28 (gastric cancer cell), HCT116 (human colorectal cancer cell), and MCF-7 cells with IC₅₀ values in the range of 0.93–2.60 µmol/L [37]. Dhinakaran and Lipton [38] displayed the antiproliferative effects of the methanol extracts of *H. atra* against HeLa (IC₅₀ = 468.0) and MCF-7 (IC₅₀ = 352.0) cell lines. Another study demonstrated that the novel active compound identified as nobilide D from *H. nobilis* exhibited inhibitory effects on K562 (human leukemic), U937 (human leukemia), A549, HeLa, MCF-7,

and HepG2 (human liver carcinoma) cell lines [39]. Kareh et al. [40] reported that the ethanolic extract and partially purified aqueous fraction of *H. polii* significantly reduced the proliferation of human mammary adenocarcinoma cells (MDA-MB-231). These data supported the view that sea cucumbers should be considered as sources of anticancer agents, and the potential of their extracts may be different according to the solvent chosen, the methods followed, the cells tested, and also the species analyzed.

Cancer can occur when the balance between cell division and cell death is disrupted and so there may be an increase in cell proliferation and a decrease in cell death [41]. Additionally, too little apoptosis has been known to occur in cancer cells [42]. Induction of apoptosis, known as a form of programmed cell death, is considered to be a significant process in controlling the progression of cancer during cancer treatment [43]. Here, the Annexin V-FITC/PI assay was used to detect the percentages of apoptotic A549 and HeLa cells after treatment with the HTM and HTS extracts at concentrations of 500, 250, and 125 µg/mL for different incubation periods. Our results showed that the HTS and HTM extracts were able to provide significant enhancements in the rates of apoptotic cells. The late stage of apoptosis was usually predominant at 48 h. The increase in the percentage of necrotic cells in the course of time may be due to the progression of apoptotic cells into secondary necrotic cells. Secondary necrotic cells are cells that have gone through various modifications during the process of apoptosis [44]. Because of the lack of phagocytic capacity, observation of secondary necrosis is frequently considered as an in vitro artifact [45]. In a previous study, Baharara et al. [46] stated that the aqueous extract of the sea cucumber species *H. arenicola* from the Persian Gulf induced apoptosis. Sangpairoj et al. [47] reported that the extract of *H. scabra* induced apoptosis, loss of mitochondrial membrane potentials, and nuclear condensation and fragmentation. The novel active compound identified as nobiliside D (at 0.5 µg/mL) from *H. nobilis* increased the rate of apoptosis in K562 and MCF-7 cells in comparison to control cells [39]. In another study, human breast ductal carcinoma cell (T47D) populations were reported to have undergone apoptosis after being treated with the ethanol extract of *H. atra* [48]. However, for the first time, the results in this study demonstrated that the mechanism underlying the cytotoxicity of both the HTS and HTM extracts may have involved induction of apoptosis in the A549 and HeLa cells.

DNA fragmentation by endogenous DNases is considered to be a hallmark of apoptosis. This may be detected through agarose gel electrophoresis because DNA fragmentation creates a ladder pattern with fragments of 180–200 base pairs in length [49,50]. Here, the apoptosis in the highest concentration (500 µg/mL) may occur more

rapidly, and this may explain why the DNA ladder might not be seen. However, our results showed that, at 250 µg/mL, the HTS and HTM extracts produced a typical DNA ladder in the A549 cells. Furthermore, DNA fragmentation was observed in the HeLa cells. These results supported the expectation that both extracts were capable of causing induction of apoptosis in the A549 and HeLa cells.

Caspase-3 is known to be one of the essential proteins associated with the apoptotic process [51] and, thus, it may be used as a marker for apoptosis. Our results indicated the presence of the highest caspase-3 activity in the cells (A549 and HeLa) treated with both extracts at 500 µg/mL in comparison to the control groups. Additionally, at 250 µg/mL, the HTS extract was found to have caused relatively more caspase-3 activation than the HTM extract. Nursid et al. [48] showed that the ethanol extract of *H. atra* activated caspase-3 on T47D cells. In the melanoma cancer cell line B16F10, the enzymatic activities of caspase-3 (as an apoptosis inducer) and caspase-9 (as an apoptosis effector) were reported to have increased among the groups treated with sea cucumber extract from *Holothuria arenicola* by Baharara et al. [52]. All of these findings confirm that extracts from sea cucumbers are capable of causing significant cytotoxicity on different cancer cell lines through apoptosis.

Important results were presented about the effects of phenolic compounds and their derivatives on cancer treatment and prevention [53]. Finally, HPLC analysis was performed to quantify the phenolic compounds in the extracts. The most abundant phenolic compounds in both the HTS and HTM extracts were detected as gallic acid, 2,5 dihydroxybenzoic acid, and ellagic acid. Remarkably, epicatechin was determined at the highest levels in the HTS extract, whereas it was only present in small amounts in the HTM extract. Moreover, the amounts of 3,4 dihydroxybenzoic acid and ellagic acid were higher in the HTS extract than in the HTM extract. *p*-coumaric acid was only detected in the HTS extract. As noted in different previous studies, the results of HPLC support the fact that the types of solvents used can alter the content and amounts of phenolic compounds according to their polarity [54,55]. It was previously reported that gallic acid has several important properties including antioxidant, antiinflammatory, and antineoplastic activities and has therapeutic effects for gastrointestinal, neuropsychological, metabolic, and cardiovascular diseases [56]. Gentisic acid or 2,5-dihydroxybenzoic acid was reported to have various biological benefits such as antiinflammatory, antirheumatic, and antioxidant effects [57], several beneficial effects against heart attacks, atherogenesis, and lipid hydroperoxide production and is able to inhibit tumor growth as a fibroblast growth factor inhibitor [58].

Likewise, ellagic acid, a natural phenolic compound, was reported to possess antioxidant, anticarcinogenic, and antiinflammatory activity [59]. Epicatechin, with its strong antioxidant properties, is a natural flavonoid and has the potential for the treatment of diabetes and cancer [60]. For this reason, our study suggested that the phenolic compounds of the extracts might contribute to their cytotoxic effects.

In conclusion, this is the first study to present the potential cytotoxic properties of *H. tubulosa* from Muğla, Turkey. Our results revealed that the aqueous and methanolic extracts of this species are capable of causing inhibition of the growth of cancer cell lines and of inducing apoptosis in A549 and HeLa cells. The HTS extract, in which epicatechin and ellagic acid were especially abundant, was rich in the contents and amounts of phenolic compounds associated with bioactivity in comparison to the HTM extract. Our study provides new details on the evaluation of *H. tubulosa* as a promising source for natural anticancer agents. Further studies may determine the biologically effective compounds from these extracts and explain the involvement of these compounds in cancer treatment. Our

studies can also be supported with different extracts of *H. tubulosa*.

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