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The cytotoxic activity of Sponges and Tunicates from Turkish Aegean Sea

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The enormous bioactive components of different species from marine habitats make them attractive candidates for the discovery of new therapeutic active substances in several diseases such as cancer. Sea sponge and tunicate materials are the ideal sources of new chemotherapeutics for various cancers due to their rich metabolites. The fundamental purpose of the current study is to investigate the cytotoxic activity of methanolic crude extracts of sponges (*Agelas oroides* and *Cladocora caespitosa*) and tunicates (*Ascidiella aspersa* and *Styela clava*) collected from the Aegean Sea. The cytotoxic activity and the anti-cancer activity of the extracts was carried out by sodium 3´- [1- (diphenyl aminocarbonyl)-3,4-tetrazolium] -bis (4-methoxy 6-nitro) benzene sulfonic acid hydrate (XTT) on gastric adenocarcinoma cell line AGS, prostate cancer cell line PC-3, neuroblastoma cell line SH-SY5Y, and mouse fibroblast cell line L929. The extracts of sponge and tunicate were found to cause different cytotoxic effects on different cancer cells, largely in a concentration-dependent manner. It is found that the extracts exhibited high anti-cancer activities in neuroblastoma and prostate cancer cell lines at concentrations of 3 and 5 mg/ml. No significant cytotoxic activity was found in L929 cells. In addition, the microscopic examination of cells treated with the extracts shows that the extracts cause morphological changes with cellular rounding, which could be associated with apoptosis.

[Keywords: Anti-cancer activity, Cytotoxic activity, Marine natural products, Sea sponge, Tunicate]

Introduction

Seas and oceans comprise of a wide diversity of species with biologically active secondary metabolites that have been proven to be a rich and promising source of new and novel bioactive natural products with potential pharmaceutical significance[1].

During the search for new drug sources for the treatment of cancer, marine natural compounds have been found to play an important role in directing drug discovery research. For this reason, the research studies in this area have been increasing extensively in recent years. Cancer is the most predominant disease in the world in terms of health economics and mortality statistics[2]. It is an extremely serious disease that killed 10 million people worldwide in 2020[ref. 3]. Today, many natural and synthetic compounds are used against different types of cancer, but the desired stage in cancer treatment has not yet been reached.

Marine sources act differently against many types of cancer, including breast and colon cancer, such as cell proliferation induction of Reactive Oxygen Species (ROS) production, mitochondrial dysfunction, Endoplasmic Reticulum (ER) stress and apoptosis. Among the marine resources, sea sponges are the most studied group, and in the last 10 years, about 100 substances have been found to have anticancer activity. Drugs like Macrolide, Eribulin Mesylate (Halaven) was obtained from sponge *Lissodendoryx* sp.[4], Gemcitabine from sponge *Tectitethya crypta*[5] and Cytarabine (Ara-C) from sponge *Cryptotheca crypta*[6] approved by Food and Drug Administration (FDA) as anticancer compound. A polyketide, Plocabulin obtained from sponge *Lithoplocamia lithistoides* (in clinical pipeline under phase II)[7] and macrolide, MORAb-202 obtained from sponge *Halichondria okadai* (in clinical pipeline under phase I)[8] are identified as anticancer compounds.

Marine sponges belonging to the *Porifera* phylum exhibit a rich profile in terms of secondary metabolites[9]. Marine sponges are of interest to researchers in the discovery of secondary metabolites. Reasons such as lack of a physical protective structure, mobility and immune system may play a role in the production of different secondary metabolites[10]. Various and unique compounds derived from sea sponges, such as

nucleosides, sterols, alkaloids and amino acid derivatives have been isolated, and at least 60 of them have indicated to have potential chemo-preventive and anticancer activities[11,12].

Similarly, tunicates are the producers of important marine natural products (mainly alkaloids and peptides) with varied levels of bioactivities such as anti-cancer, anti-microbial, anti-fouling, anti-deterrent and anti-viral[13]. A total of three tunicate-derived compounds are in clinical use. Trabectedin (Yondelis®) isolated from *Ecteinascidia turbinate*, is currently in use to treat soft-tissue sarcoma and ovarian cancer[14]. Lurbinectedin (aka Zepzelca®) is a synthetic derivative of trabectedin that has demonstrated to have a substantially higher tolerated dose than its natural product counterpart[15], and high survival rate in Phase III clinical trials in ovarian cancer. Plitidepsin (aka Aplidin® dehydrodidemnin B) was originally isolated from *Aplidium albicans*, has been certified in Australia for the use against multiple myeloma[16].

In the United States, there are thirteen FDA approved marine-derived drugs[17]. Currently, Plitidepsin (Aplidin®) has been approved by Australia Therapeutic Goods Administration (TGA) and is starting Phase III studies for the treatment of COVID-19[ref. 18]. 40 compounds isolated from marine organisms are currently undergoing clinical trials at different phases[19].

Considering the high biological potentials of sea sponges and tunicates, the aim of this study is to investigate the anti-cancer effect of methanolic raw/crude extracts of sponge (*Agelas oroides* and *Cladocora caespitosa*) and tunicate species (*Ascidiella aspersa* and *Styela clava*) from the Aegean Sea on various cancer cell lines.

Material and Methods

Materials examined

Sponge material (*Agelas oroides* (Schmidt, 1864) and *Cladocora caespitosa* (Linnaeus, 1767)) and tunicate material (*Ascidiella aspersa* (Müller, 1776) and *Styela clava* Herdman, 1881) were collected by scuba-diving from the Aegean Sea (Kömür Limanı, depth: 20 m) in August 2018. The collected specimens were identified by Dr. Bülent Gözcelioğlu. A voucher specimen of sponges and tunicates are deposited at the Pharmacognosy Department of Faculty of Pharmacy, Ankara University.

Sample preparation

The sponge and tunicate materials were ground and extracted with methanol (500 mL) for four times. Obtained methanol phase were filtered and evaporated in vacuum until dryness. The obtained dry methanol extract was preserved at 4 °C until its use. Buchi R-300 (Switzerland) rotary evaporator systems were used to evaporate solvents and obtain dry crude extracts.

Cell culture

The human gastric adenocarcinoma cell line AGS, prostate cancer cell line PC-3, neuroblastoma cell line SH-SY5Y and mouse fibroblast cell line L929 were used for assessing anticancer activity at different concentrations. Cells were cultured at 37 °C under a humidified atmosphere and 5 % CO_2 in DMEM-F12 medium supplemented with 10 % fetal bovine serum and 1 % pen/strep. Cells reaching 70 – 80 % confluency were detached from the surface with 0.25 % (w/v) trypsin / EDTA and used for assays[20].

Anticancer and cytotoxicity assays

The cytotoxic effects of the sponge and tunicate extracts were investigated on L929 mouse fibroblast cells as a normal cell line. Anticancer activity was evaluated on three different human cancer cell lines, AGS, SHSY5Y, and PC-3. The cytotoxic and anticancer activity of the extracts was examined using XTT (sodium 3´- [1- (diphenyl aminocarbonyl)-3,4-tetrazolium] -bis (4-methoxy 6-nitro) benzene sulfonic acid hydrate) cell viability assay[20].

Briefly, a stock solution of 10 mg/mL in culture medium was prepared for each test sample and the solutions were filtered 0.45 µm for sterilization and to remove insoluble matter. The cells were seeded in 96 well plates at a concentration of 10^4 cells/well for cell viability assays. After 24 h incubation, the medium was removed and replaced by 100 µL fresh medium containing the samples and cells were incubated for 24 h at 37 °C. After 24 h of incubation with the samples, the medium was replaced with a medium containing 0,4 mg/ml XTT and incubated for 4 h and absorbance values of the wells were recorded at 450 nm in microplate reader and cell viability was calculated using the formula[20]:

$$Viability = \left(\frac{Optical\ density\ (OD)\ of\ treated\ cells}{OD\ of\ control\ cells}\right) x100$$

Results

In this study, human stomach (AGS), prostate (PC-3) and neuroblastoma (SH-SY5Y) cancer cells were selected to determine the anticancer activities of sponge (*A. oroides* and *C. caespitosa*) and tunicate

(*A. aspersa* and *S. clava*) material extracts. In addition, the cytotoxic effect of the extracts was tested on normal mouse fibroblasts (L929). For cytotoxic activity, working extract concentrations range used were of 1.5 – 5 mg/ml. In addition, microscopic images were obtained to observe changes in cell morphology after 24 h of samples treatment.

Cytotoxic activity of sponge and tunicate extracts on L929 cells

Before examining the anticancer effects of the extracts in three different cancer cell lines, their cytotoxic effects in the mouse fibroblast L929 cell line were investigated. The results of the analysis are shown in Figure 1. The samples induced different effects on fibroblast cells at different concentrations (Fig. 2). The cell viability for *A. oroides* has decreased compared to the control with increase in extract concentration, whereas cell viability with *C. caespitosa*, *A. aspersa*, and *S. clava* extract did not show concentration-dependent decrease or increase. However, cell viability at above 70 % of all concentrations for all extracts indicates that the samples show high biocompatibility on mouse fibroblast cells.

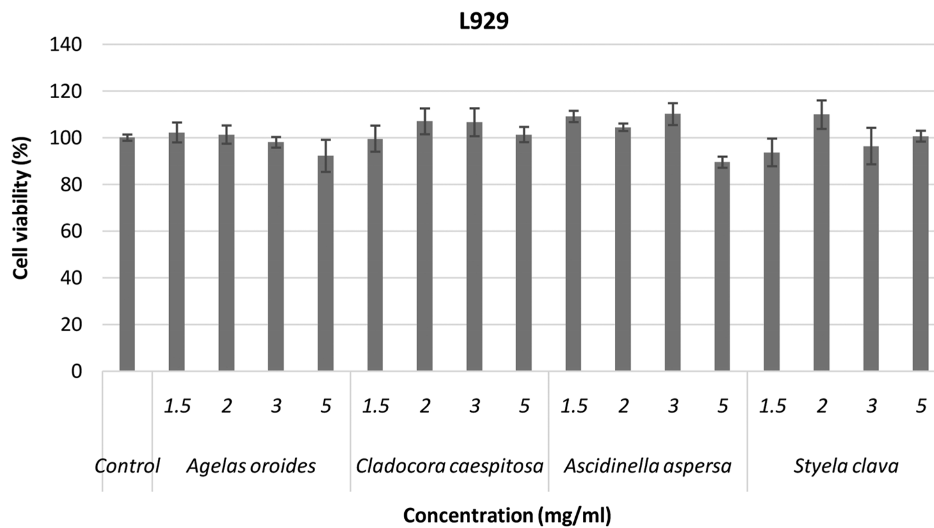

Fig. 1 — Cell viability of L929 cell line with various extract concentrations treatment for 24 h

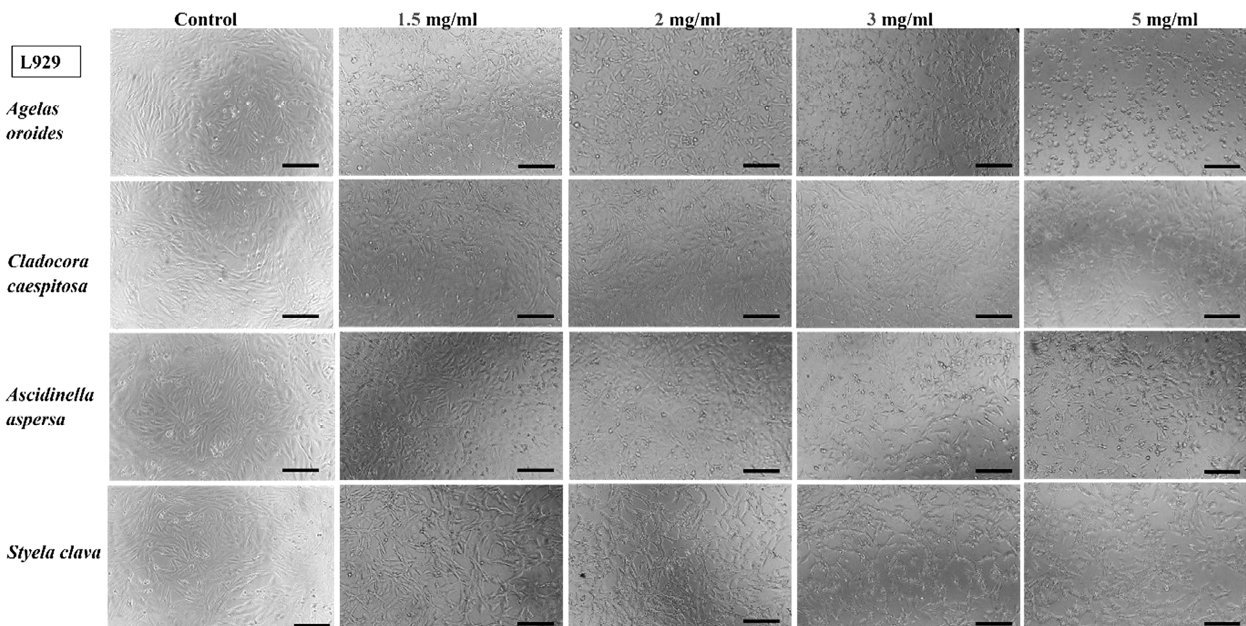

Fig. 2 — Microscopic images of L929 cells treated with extracts for 24 h *(Scale bar: 200 µm)*

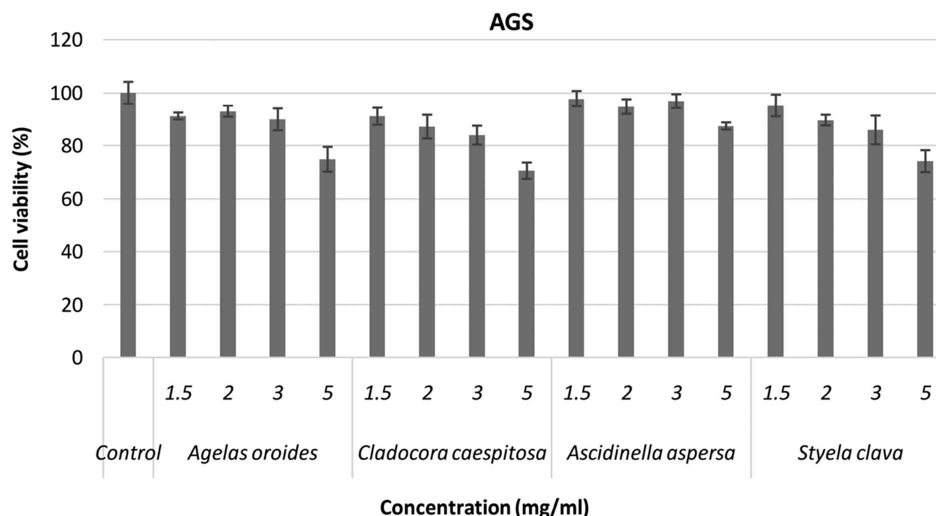

Fig. 3 — Cell viability of AGS cell line with various extract concentrations treatment for 24 h

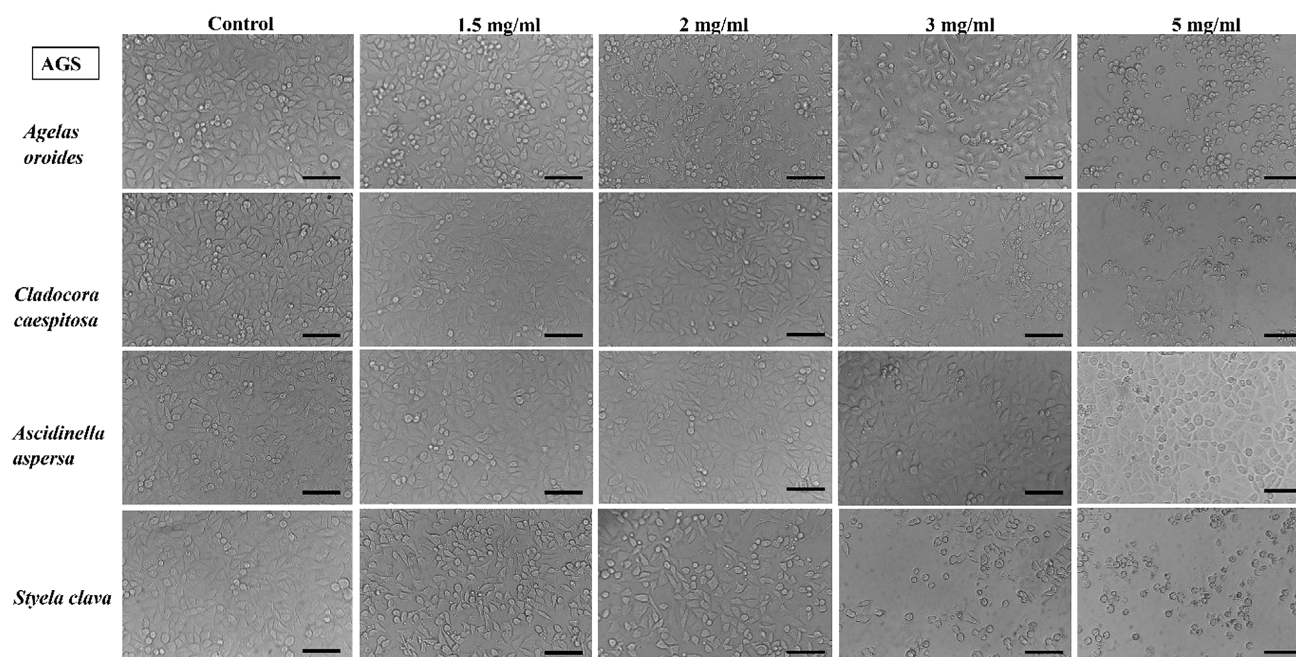

Fig. 4 — Microscopic images of AGS cells treated with extracts for 24 h *(Scale bar: 200 µm)*

Anticancer activity of sponge and tunicate material extracts on AGS cells

Figure 3 shows the cytotoxic effects of the different concentrations of each extract on the AGS cell line. The results revealed that all extracts decrease the cell viability of AGS cells in a concentration-dependent manner compared to control. Microscopic examination of AGS cells presented at Figure 4. However, for all extracts, cytotoxic activity on AGS cells was less than other cancer cell lines. Even at high concentrations, cell viability was above 70 % and the lowest cell viability was for *C. caespitosa*. The lowest cell viability for *A. oroides*, *C. caespitosa*, *A. aspersa*, and *S. clava* were 74.9±4.6, 70.6±3, 87.4±1.3, and 74.2±4 %, respectively at 5 mg/ml concentrations. *A. aspersa* was relatively ineffective in AGS cells compared to other extracts. The results from the *S. clava* extract were similar to the previously reported anticancer activity of different hydrolysates in AGS cells, but as expected for higher extract concentrations[21].

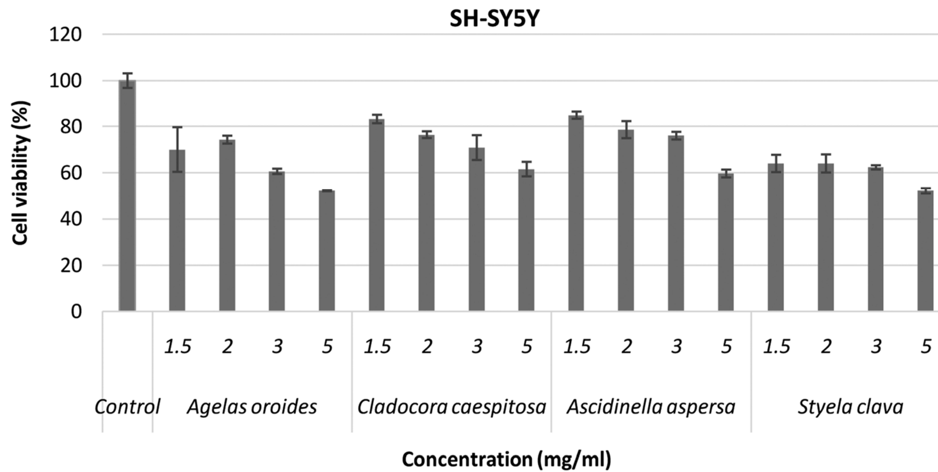

Fig. 5 — Cell viability of SH-SY5Y cell line with various extract concentrations treatment for 24 h

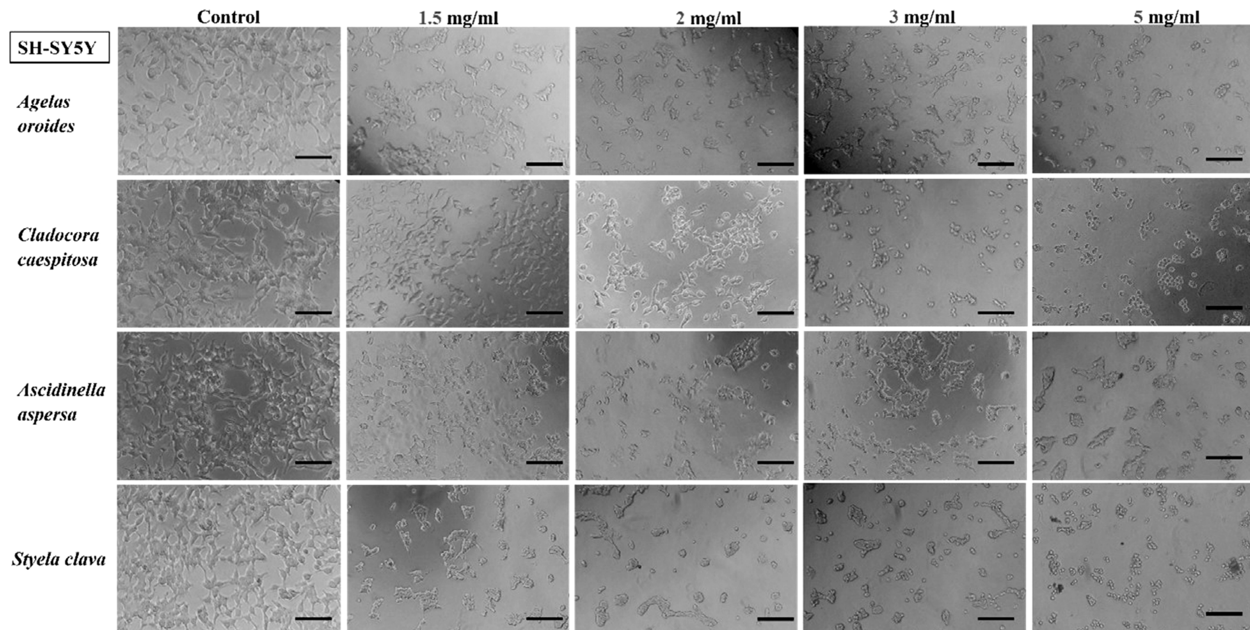

Fig. 6 — Microscopic images of SH-SY5Y cells treated with extracts for 24 h *(Scale bar: 200 µm)*

Anticancer activity of sponge and tunicate material extracts on SH-SY5Y cells

Figure 5 shows the cytotoxic effects of the extracts on SH-SY5Y cells. Cell viability decreased for each extract in a concentration-dependent manner for SH-SY5Y cells as with AGS. However, all extracts induced higher anticancer activity characterized by low cell viability for neuroblastoma cells compared to AGS cells. In SH-SY5Y cells, cellular morphological changes were easier to spot (Fig. 6). *C. caespitosa* and *A. aspersa* had similar effects on SH-SY5Y cells and resulted in reduced cell viability at concentrations of 5 mg/ml. The lowest cell viability for *A. oroides* and *S. clava* were 52.1±0.2 and 52.1±1.1 %, respectively at 5 mg/ml concentrations. The extract in which the increase in concentration affected the cell viability the least was *S. clava*. However, *A. oroides* and *S. clava* have exhibited high cytotoxic activity even at lower concentrations (3 mg/ml). It has been previously reported that *A. oroides* could induce caspase-dependent apoptosis associated with oxidative stress in neuroblastoma cells, even at concentrations of 10 and 20 µg/ml[22].

Anticancer activity of sponge and tunicate material extracts on PC-3 cells

The cytotoxic effects of the extracts on PC-3 cells are shown in Figure 7. As with AGS and SH-SY5Y cells, the extracts led to a concentration-dependent

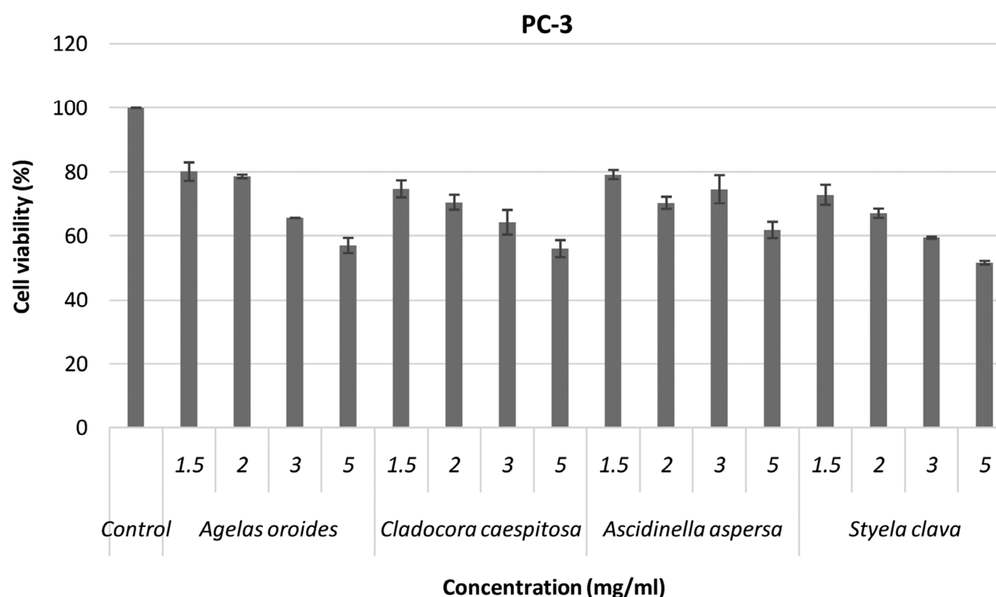

Fig. 7 — Cell viability of PC-3 cell line with various extract concentrations treatment for 24 h

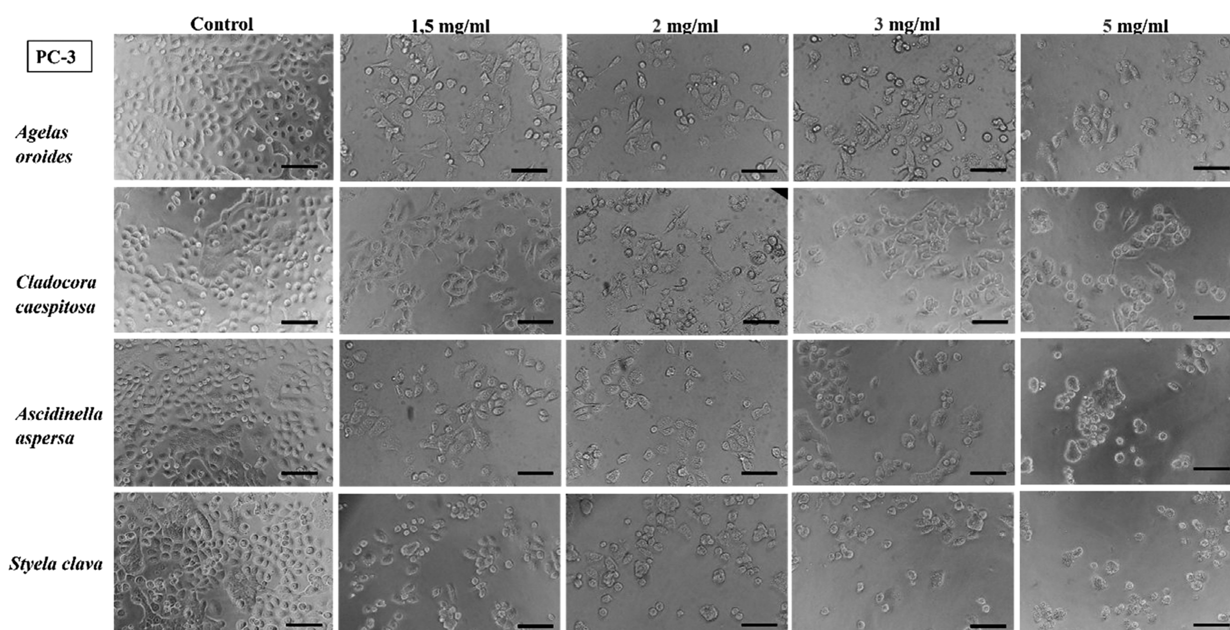

Fig. 8 — Microscopic images of PC-3 cells treated with extracts for 24 h *(Scale bar: 100 µm)*

decrease in cell viability in PC-3 cells (except 3 mg/ml concentration in *A. aspersa*). Microscopic examination of PC-3 cells demonstrated cellular rounding and reductions in cell population (Fig. 8). Cell viability at 5 mg/ml concentrations for samples *A. oroides*, *C. caespitosa*, *A. aspersa*, and *S. clava* were 56.9±2.9, 56±3.2, 61.8±3.1 and 51.6±0.7 %, respectively. All concentrations of *S. clava* have resulted in higher cytotoxicity compared to other extracts.

Microscopic examination of cell cultures

In the cell cultures treated with various chemical or biological agents, different cellular changes such as morphological changes, loss of adhesion, cell death, and fragmentation are highly associated with cytotoxic effects. It has also been reported in the previous studies that some sponge species cause changes in cell morphology and microtubule organization[23,24]. Therefore, in this study, microscopic images of cells

treated with extracts for 24 h were compared with control groups and cellular morphological changes were observed for each extract at its varying concentration. Control cells in all cell groups had a regular shape without any morphological changes. However, rounding and separation due to the increase in concentration were observed in the groups treated with the sponge and tunicate extracts. The size of the cell-free areas in AGS cells was remarkable at 3 mg/ml concentrations of *S. clava* extract (Fig. 4). Although the cells lost their characteristic shape to a large extent at 5 mg/ml concentrations, the fact that the XTT result (cell viability was 74.2 % at 5 mg/ml) was above 70 % suggested that the 24-hour incubation period could be extended for more pronounced cytotoxic effects in AGS cells (Fig. 3). In SH-SY5Y cells, cellular morphological changes were more evident even at low concentrations. Cellular aggregates observed in *A. oroides* and *S. clava* cultures at 1.5 mg/ml were notable (Fig. 6). Rounding and cell-free areas were observed at higher concentrations. It has been previously reported that *A. oroides* extract could cause morphological changes by specifically affecting cytoskeletal proteins in neuroblastoma cells at low concentrations[22]. However, it should be considered that these morphological changes may result also from cellular differentiation[25]. Microscopic examination of PC-3 cells shows that cellular rounding and reductions in cell population occur even at extract concentrations of 1.5 mg/ml, as in SH-SY5Y cells (Fig. 8).

In summary, sponge, and tunicate extract-induced cytotoxicity in three different cancer cell lines played a role in morphological changes, and these changes seem to be consistent with toxicity results. Besides, extracts at different concentrations did not lead to significant changes in cell morphologies in L929 cells (Fig. 2), again consistent with cytotoxicity results. The results presented in this study are remarkable considering that cytotoxicity-induced morphological changes caused by the sponge and tunicate extracts that were used in the present study in the cancer cell lines have not been examined before.

Discussion

In the present study, the *in-vitro* cytotoxic activity of sponge (*A. oroides* and *C. caespitosa*) and tunicate (*A. aspersa* and *S. clava*) material extracts for three different human cancer cells and mouse normal cells have been evaluated. The results have shown high cytotoxic effects of the extracts, which may be associated with anticancer activity. Moreover, the fact that the extracts did not show a significant cytotoxic effect on L929 cells suggests that this cytotoxic effect could be selective for cancer cells. However, it is thought that more studies should be conducted to determine the precise anticancer activity and anticancer mechanisms. Also, the results are remarkable in the literature as it is the first study investigating the cytotoxic effects of *C. caespitosa* and *A. aspersa* on different cancer cells.

Conclusion

The therapeutic and commercial use of marine anticancer substances depends on preclinical and clinical studies. Currently, many marine natural products have been reported to be in the process of development for drugs. Therefore, it is necessary to study the anticancer activities of marine sources in order to develop novel anticancer drugs for diverse cancers.

Although Turkey has approximately 8400 km of coastline, there are very few studies on marine secondary metabolites and bioactivities of these resources. Therefore, the results obtained from this study could be a source for the future studies. Our hope is to motivate efforts to continue searching the sea for the antitumor compounds.

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Conflict of Interest

Authors declare there is no conflict of interest.

Ethical statement

The authors declare this article does not contain any studies involving human participants or animals performed by any of the authors.

Author Contributions

BK & ISU: Conceptualization, resources, provision of study materials, and writing - review & editing. BM: Visualization, and data presentation. RÇK: Conceptualization, methodology, formal analysis, anticancer and cytotoxic assays. RÇK & BM: Writing - original draft.

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