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Full Length Research Paper

Assessment of *in vitro* antitumoral and antimicrobial activities of marine algae harvested from the eastern Mediterranean sea

E. Taskin*, Z. Caki, M. Ozturk and E. Taskin

Department of Biology, Faculty of Arts and Sciences, Celal Bayar University, Muradiye-Manisa 45140, Turkey.

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Antitumoral activities of five algal extracts obtained from the marine algae *Scytosiphon lomentaria, Padina pavonica, Cystoseira mediterranea (Phaeophyceae), Hypnea musciformis* and *Spyridia filamentosa* (Rhodophyta) were assessed against the human breast adenocarcinoma cell line MCF–7 and the human prostate carcinoma epithelium like cell lines DU 145, LNCaP, PC3 using the cytotoxic assay, *in vitro*. The crude extract of *S. filamentosa* showed strong cytotoxic activity against the DU-145 cell line, and it showed less than 10% cell viability after treatment. Antimicrobial activities of the crude extracts of algae (with the exception of *H. musciformis*) were also tested by disc diffusion assay against three Gram positive and five Gram negative bacterial strains and against the yeast pathogen *Candida albicans*. Among the extraxts, *S. lomentaria* extract (prepared with methanol) inhibited highly Gram negative bacterium *Salmonella typhimurium* growth while *C. albicans* growth was only inhibited by *C. mediterrranea* extract.

Key words: Antimicrobial activity, Mediterranean sea, *in vitro* cytotoxicity, marine algae.

INTRODUCTION

Marine algae are one of the largest producers of biomass in the marine environments (Bhadury and Wright, 2004). They produce a wide variety of chemically active metabolites in their surroundings, potentially as an aid to protect themselves against the other settling organisms. These active metabolites also known as biogenic compounds, such as halogenated compounds, alcohols, aldehydes, terpenoids are produced by several species of marine macro and microalgae and have antibacterial, antialgal, antimacrofouling and antifungal properties which are effective in the prevention of biofouling and have other likely uses, e.g. in therapeutics (Bhadury and Wright, 2004; Smit, 2004).

Another area is the search for anticancer drugs since marine molecules particularly obtained from marine invertebrates have led to promising results in trials at different phases of cancer diseases (Mayer and Gustafson, 2006). Great part of these molecules was obtained from some marine sponges, bryozoan and mollusca. Numerous macroalgae have shown potent cytotoxic activities and certain authors have suggested the consumtion of algae as a chemo-preventive agent against several cancers. Dehydro-thrsiferol and halomon extracted from *Laurencia viridis* Gil-Rodriguez et Haroun and *Portieria hornemanii* (Lyngbye) P.C. Silva, respectively have been tested in the preclinical phase. Concerning brown algae, polysaccharides and terpenoids are considered as promising bioactive molecules in the search for anticancer drugs (Zubia et al., 2009). Marine algae are also rich sources of unsaturated fatty acids and these fatty acids were also reported to block growth and systemic spread of human breast cancer via mechanisms independent of the host immune system, perhaps by peroxidation of intracellular lipids (Devery et al., 2001). Extracts of the Fijian green alga *Tydemania expeditionis* Weber-van Bosse and of the red alga *Hydrolithon reinboldii* (Weber-Van Bosse et Foslie) Foslie were found to be potentially cytotoxic in an invertebrate toxicity assay. Novel fatty acids and lipids

*Corresponding author. E-mail: evrim.taskin@bayar.edu.tr.

Abbreviations: DMSO, Dimethlysulfoxide; **FBS,** fetal bovine serum; **PBS,** phosphate-buffered saline.

were isolated and elucidated structurally from these two species, recently (Wan-Jiang et al., 2008). Halogenated compounds are produced mainly from marine red and brown algae and these compounds are dispersed in several different classes of primary and secondary metabolites, including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons. These were reported as having biological activites including antibacterial and antitumoral (Cardozo et al., 2007).

Several metabolites obtained from algae were identified and shown to possess cytotoxic properties. Some of their mechanism of action were elucidated such as kahahalide F acting on lysosomal membrane and inducting cell necrosis, several sulphated macroalgal polysaccharides (e.g.fucoidans, translam, ulvan) reducing cell proliferation and terpenes (e.g. caulerpenynes from *Caulerpa taxifolia*, mediterraneol from *Cystoseira mediterranea*, meroterpenes and usneoidone E and Z from *Cystophora usneoides*) inhibiting mitotic cell division (Smit, 2004). Several antitumor compounds from algae such as halomon had been developed to the clinical phase (Egorin et al., 1996).

Bioactive compounds with antimicrobial activity from marine macro algae have also been studied extensively. Antimicrobial substances secreted by algae have been observed. Numerous studies are being carried out globally in order to check the bioactivities of algae or to find out compounds for different purposes such as pharmaceutical, cosmetics and food preservatives, antifouling coatings, etc. (Sastry and Rao, 1994; Armstrong et al., 2000; Gonzalez et al., 2001; Salvador et al., 2007).

With almost 700 identified macro algae species, the coasts of Turkey are rich in macroalgae (Taskin et al., 2008). The marine algae flora of North Cyprus was studied by Ozturk et al. (2008) and about 300 species were reported. Some of these marine algae were studied for their different bioactivities in recent years (Salvador et al., 2007; Ozdemir et al., 2006; Tuney et al., 2006; Taskin et al., 2007).

In this study crude extracts of five marine algae three of which belong to Phaeophyceae [*Scytosiphon lomentaria* (Lyngbye) Link*, Padina pavonica* (L.) Thivy*, C. mediterranea* Sauvageau) and two of which belong to Rhodophyta [*Spyridia filamentosa* (Wulfen) Harvey*, Hypnea musciformis* (Wulfen) J.V. Lamouroux] collected from the Eastern Mediterranean Sea were studied for their *in vitro* antitumoral and four of them for antimicrobial activities since these algae groups were determined as containing bioactive compounds, in literature.

MATERIALS AND METHODS

Algal sampling and classification

Seaweeds were collected by scuba diving from three stations along the coastline of the Mediterranean coast of Turkey and North Cyprus (NC). Five algal species four of which were from Ayvalik and Çanakkale coastline (Turkey) and *S. filamentosa* from NC were collected. Algae were taken to the laboratory on ice immediately after harvesting and kept frozen at -20°C until further use. Algae samples were identified by Assoc. Prof. Ergün Taskin (Celal Bayar University, Faculty of Arts and Sciences, Biology Department, Manisa, Turkey) according to Fletcher (1987), Gómez-Garreta et al. (2001) and Maggs and Hommersand (1993). The voucher specimens were deposited at his personal herbarium.

Preparation of algal extracts

Algae samples were washed twice with distilled water and air-dried on a blotting paper. Ten grams of milled algal species were added to 150 ml of methanol and left for 8 h at room temperature and stirring at 200 rpm. The solvent extracts were then filtered and the filtrate was concentrated by rotary evaporation at 45 - 50°C. The resulting extracts were then dissolved in dimethlysulfoxide (DMSO) and kept at +4°C until further use.

Cell lines and culture conditions

MCF-7 cells were kindly provided by the Ministry of Agriculture and Rural Affairs Alum Institute and other cells were kindly provided by Assoc. Prof. Kemal S. Korkmaz (Ege University, Engineering Faculty, Department of Bioengineering). Cells were cultured in RPMI1640 medium (Gibco, Invitrogen, US), supplemented with 10% fetal bovine serum (FBS), 10.000 unit ml^{-1} penicillin G (Gibco, Invitrogen, US), 10 mg ml^{-1} streptomycin (Gibco, Invitrogen, US) and 1% nonessential aminoacids (Sigma Chemicals Co.,St. Louis, USA). Plates were incubated for 3 - 4 days at 37°C 5% CO_2 incubator (Thermo, Fisher Scientific, USA).

In vitro cytotoxic actvity

The cytotoxic activities of the five algal crude extracts were examined using colorimetric 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT assay (Mossmann, 1983). This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. The resulting purple solution is spectrophotometrically measured.

The cells which were kept in the liquid nitrogen (-196°C) were thawed at 37°C and cultured as defined before. Algal extracts (0.1 - 0.4%) were dissolved in DMSO as a universal solvent to dissolve the crude extracts and then filtred through a sterile millipore filter (0.22 µm).

Extracts were diluted with phosphate-buffered saline (PBS) solution in 1:10. The serial dilutions of the extracts were seeded in 96-well microtiter plates and the cells were added onto the extracts and then, incubated. The inhibition of growth rate of cell lines (MCF-7, DU 145, LNCaP, PC3) was noted at 24, 48 and 72 h. Before the end of the incubation period, medium of the cells was removed and wells were washed by pre-warmed PBS to remove any trace of compounds and to prevent color interference during optical density determination. MTT stock-solution (5 mg ml^{-1}) was diluted at 1:10 ratio into complete culture media, 100 µl of MTT dilution was added into each well and incubated at 37°C in humidified atmosphere. After 4 h, plates were centrifuged at 1800 rpm for 10 min at room temperature to avoid accidental removal of formazan crystals. Crystals were dissolved with 100 µl DMSO. The absorbance was determined at 540 nm. Results were represented as percentage cell viability. Assays were run in duplicate and the average results were given.

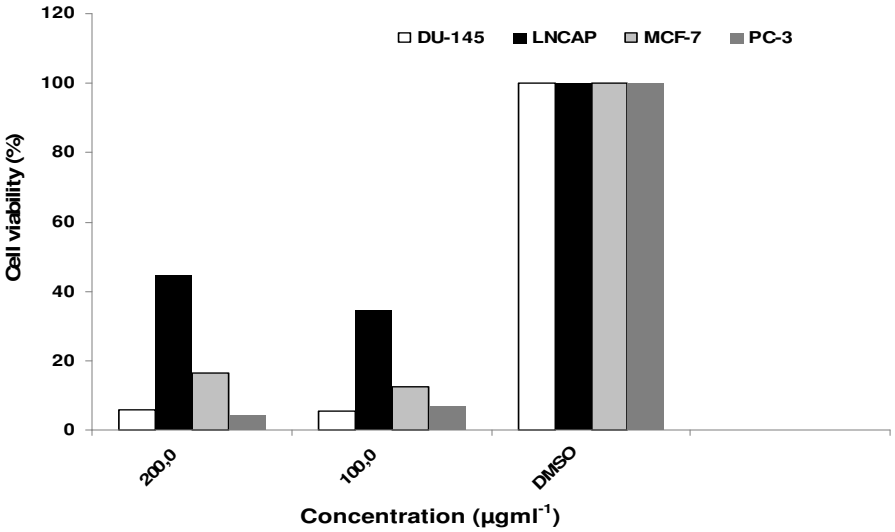

Figure 1. Cytotoxic activities of crude extracts in 100 and 200 µg ml^{-1} concentrations prepared from *S. filamentosa* against the four tumor cell lines (24 h incubation period).

Antimicrobial assay

The algal extracts described earlier were also used for the anti-bacterial assays using the paper disc diffusion method (El-Masry et al., 2000). Test microorganisms were cultivated on Mueller Hinton Broth (Merck, Darmstadt, Germany) at 37°C for 18 h before the inoculation for assay. 100 µl of broth culture which contains 10^7 - 10^8 numbers of bacteria per ml was added to Tryptic Soy agar (Merck, Darmstadt, Germany) medium and poured to sterile petri dishes. After the medium has solidified, the discs impregnated with extracts (60 µL per disc in 6 mm in diameter) were placed onto the surface. Dishes were incubated for 24 h at 35°C for bacteria and at 28°C for the yeast. Assays were carried out in triplicate. Discs impreganated either with methanol and DMSO was used as negative control while tobramycin and nystatin discs (10 and 30 µg/disc, respectively) were used positive control. After incubation the clearence zones around the discs were measured and expressed in milimeter. The test organisms that were used in this study and inhibition zones observed by the antibiotics are given in Figure 3.

RESULTS AND DISCUSSION

The results for *in vitro* cytotoxic activity of *S. filamentosa* extract are given in Figure 1 and for the four algal extracts are given in Figure 2. Two concentrations of all extracts as 100 and 200 µg per ml were examined for 24, 48 and 72 h incubation period except *S. filamentosa* extract which was incubated for 24h. The highest cytotoxic activity among all extracts was shown by *S. filamentosa* extract in 100 µg ml^{-1} concentration with higher than 90% cell inhibition against the DU-145 cell line and about 80% against the MCF-7 cell line. *C. mediterrranea* extract in 100 µg ml^{-1} concentration showed 55% cell inhibition against the MCF-7 cells.

S. filamentosa extracts were assessed against four tumor cell lines and the most sensitive cell line to the extract was found as DU-145, while the LN-CAP cell line was the most resistant to the extract. However, in both concentrations (100 and 200 µg ml^{-1}) of extracts, viabilites of cell lines did not exceed 50%.

P. pavonica showed good cytotoxic activities with 200 than 100 µg ml^{-1} concentrations in 24 h (higher than 80% inhibition). Ktari and Guyot (1999) reported that the dichloromethane extract of *P. pavonica* showed high cytotoxic activity against the human buccal epidermal carcinoma (KB) cells in 10 µg ml^{-1} concentration, while dichloromethane/methanol extract showed moderate inhibition (Ktari and Guyot, 1999). Cytotoxic compound was isolated and identified as oxysterol. Extraction solvent and season of collection of specimens were indicated as important for biological activity of the extracts of algae. Many side chain-oxygenated sterols have been isolated from a number of brown algae (Kerr and Baker 1991). El Masry et al. (1995) reported a high percentage of anti-tumorigenic activity against *Agro-bacterium tumefaciens* in *P. pavonica* harvested in Japan during winter, and found that the activity depends on the season of harvesting, but the product responsible for the activity was not isolated.

Zubia et al. (2009) investigated the cytotoxic activities of crude extracts from Sargassaceae and those from *Desmarestia ligulata* and *Dictyota dichotoma* showed strong cytotoxicities against the human cancer cell lines.

Various diterpenes have been identified by Zubia et al. (2009) as the bioactive compounds in several species of the genus *Cystoseira*. These diterpene compounds could be responsible for the antitumoral activities of the *Cystoseira* extracts (Zubia et al., 2009).

In this study, the results of cytotoxic activities of extracts were found more efficient in 24 h than the 48 and 72h incubation periods. On that basis, the differences

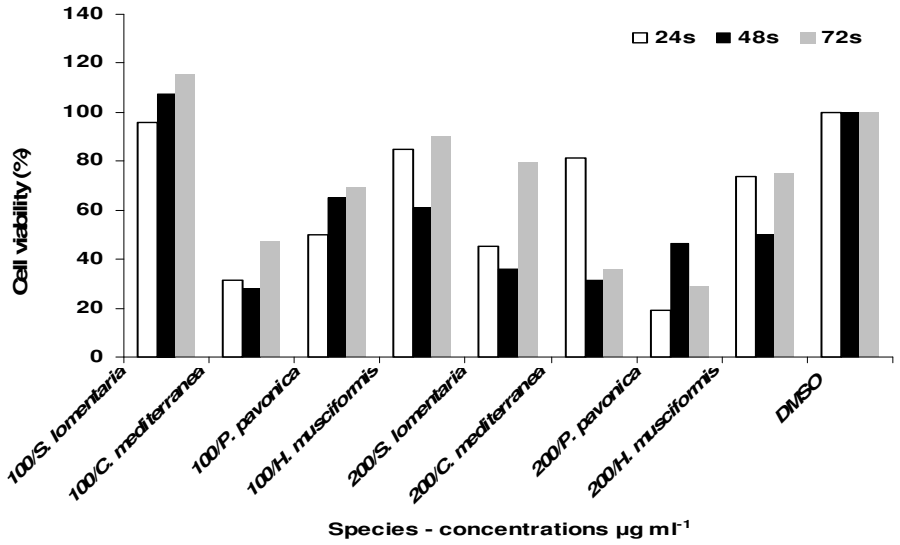

Figure 2. Cytotoxic activities of crude extracts in 100 and 200 µgml^{-1} concentrations prepared from *S. lomentaria, P. pavonica, C. mediterranea, H. musciformis* against the four tumor cell lines.

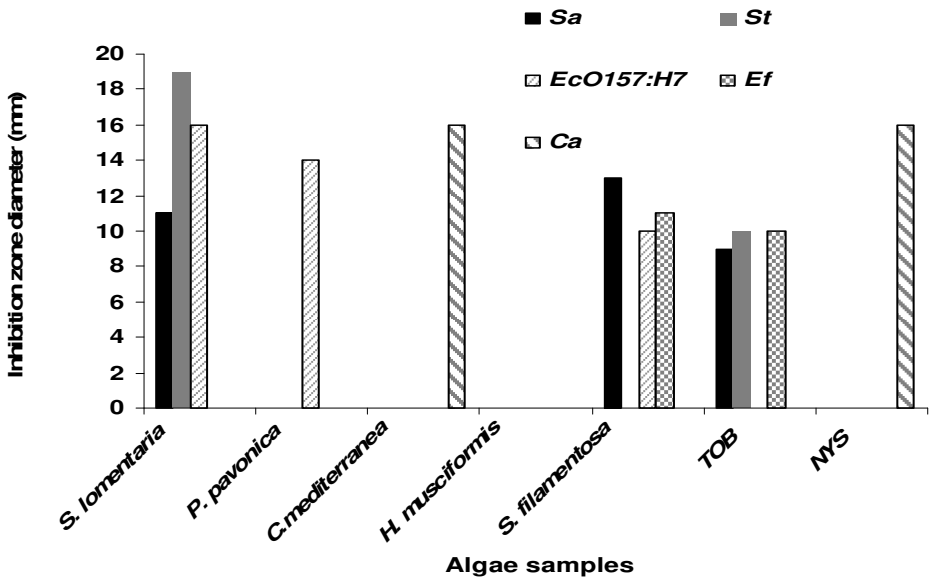

Figure 3. Inhibited test microorganisms by the crude extracts of algae and average diameters of inhibition zones. Sa: *Staphylococcus aureus*, St: *Salmonella typhimurium*, EcO: E*scherichia coli* O157:H7, Ef: *Enteroccus faecalis*, Ca: *Candida albicans*, TOB: Tobramycin, NYS: Nystatin.

between the results could be corresponded to the incubation time or whether the tumor cells proliferated after the incubation period. *H. musciformis* and *S. lomentaria* extracts in 100 µg ml^{-1} concentrations showed low toxicities against the cell lines (Figure 3). However, 200 µg ml^{-1} concentration of *S. lomentaria* extract showed higher toxicity in 48 h (<40% cell viability).

Crude or partially puified polysaccharides from various brown algae showed antitumor activities against the experimental tumor. In the report of Noda et al. (1990) the brown algae *S.lomentaria* (69.8% inhibition) and *Sargassum ringgoldianum* Harvey showed antitumor activity against implanted Ehrlich carcinoma (Noda et al., 1990). Xu et al. (2004) investigated 39 species of macroalgae from China caosts for their antitumoral activities and found that the ethanol extract of *S. lomentarius* had strong selective cytotoxic effect against KB cells (IC_{50} <4.40 µg ml^{-1}). The antitumor activities of polysaccharides

are thought to be based on the increase of phagocytosis in the reticuloendothelial system.

In another study carried on *Cystoseira tamariscifolia* (Hudson) Papenfuss samples collected from Morocco, highest cytotoxic activity was determined by CH_2Cl_2 fraction, in 20 µg ml^{-1} concentration of extract (30% cell inhibition) (Abourriche et al., 1999).

In this study, antimicrobial activities of the extracts were also assessed against some gram positive and negative bacterial strains and obtained inhibition zones (IZ) against test microorganisms are shown in Figure 3. Among the test microorganisms *E. coli* O157:H7 serotype (a foddborne pathogen) was found most sensitive against the extracts. This bacterium was mostly inhibited by *S. lomentaria* extract (16 mm IZ) and followed by *C. mediterranea, P. pavonica* and *S. filamentosa* extracts. *Salmonella typhimurium* was only and strongly inhibited by the *S. lomentaria* extract. Tuney et al. (2006) observed weak activity of *P. pavonica* ethanol extract against test microorganisms, while diethylether extract of *C. mediterranea* showed strong inhibitor activity against both Gram positive and negative bacteria. In conclusion, Gram negative bacterial strains were found more sensitive to the extracts than Gram positive bacteria and the yeast pathogen as can be seen in Figure 3.

Ballantine et al. (1987) found that the extracts prepared from several algal species including *Caulerpa verticillata* J. Agardh, *D. dichotoma* (Hudson) J.V. Lamouroux, *S. filamentosa* (Wulfen) Harvey were all found as inactive at one sampling time, while active at another sampling time against one set of test micro-organisms, whereas active against an entirely different set of microorganisms at another sampling time. These results indicate the possibility that different quantities of active secondary products were synthesized under different conditions and/or different active compounds against test microorganisms were synthesized.

In another study carried by Salvador et al. (2007) the extracts that were prepared from fresh and lyophilized samples of algae *C. mediterranea, S. lomentaria, P. pavonica* which were collected during the four seasons and *H. musciformis* which was collected only in summer seson were screened for antimicrobial activities and the actitvites were compared. Bioactivities of the extracts showed differences depending on the season that samples were collected and the type of samples which the extracts have been prepared. *C. mediterranea* extract had the broadest inhibitor activities against the test microorganisms except the extract of algae which was prepared from sample that was collected in winter (highest inhibitor activity against to *E.coli* 17.1 mm IZ in autumn). *S. lomentaria* extracts did not show inhibitory activities. *P. pavonica* extract that was prepared from samples which were collected in winter showed highest inhibitor activity (most active against to *S. aureus*, 14.1 mm = IZ) while the extract of *H. musciformis* showed inhibitory activity against only *Staphylococcus aureus* (14.3 mm = IZ).

Conclusion

In this study, the *in vitro* cytotoxic acitivites of five algal species, four of which were collected from Aegean Shores of Turkey and one of which was from North Cyprus shores were assessed for the first time for these locations. Differences among the results of the activities of extracts were observed with the studies which were carried out from other countries of the world. However, in this study, without using any seperation and fractionation steps, strong cell inhibition (90%) was obtained by the crude extract of *S. filamentosa. C. mediterranea* and *P. pavonica* extracts were also found as having reasonable cytotoxic activities against the assessed cell lines. Therefore, these three alga species were found as prominent for further cytotoxic activity studies.

Compounds responsible for antimicrobial activities are widespread in algae as shown by previous studies.

Inhibition zones for antimicrobial activities obtained by the same extracts of algae samples were in the range of moderate to strong. Differences observed by the other studies are contributed by several factors, mainly infra-specific variabilities in the production of secondary metabolites. In the next studies, collecting these studied species in different seasons and using different extraction solvents, extraction methods, seperation steps, and their cytotoxic and antimicrobial activities should be monitored.

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