Cytotoxic activity of ten algae from the Persian Gulf and Oman Sea on human breast cancer cell lines; MDA-MB-231, MCF-7, and T-47D

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ABSTRACT

Background: Seaweeds have proven to be a promising natural source of bioactive metabolites for drug development. **Objective:** This study aimed to monitor the ethanol extract of ten algae from the Persian Gulf and Oman Sea, for their *in vitro* cytotoxic activity on three human breast cancer cell lines. **Materials and Methods:** Three human breast cancer cell lines including MDA-MB-231(ER^-), MCF-7(ER^+), and T-47D (ER^+) were treated by different concentrations of total ethanol (90%) algae extracts and the cytotoxic effects were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Doxorubicin (Ebewe, Austria) was used as a positive control. After 72 h of incubation, the cytotoxic effect of the algae was calculated and presented as 50%-inhibitory concentration (IC_{50}). **Results:** The results indicated *Gracilaria foliifera* and *Cladophoropsis* sp. to be the most active algae in terms of cytotoxic effects on the investigated cancer cell lines. The IC_{50} values against MDA-MB-231, MCF-7, and T-47D cells were, respectively, 74.89 ± 21.71, 207.81 ± 12.07, and 203.25 ± 30.98 μg/ml for *G. foliifera* and 66.48 ± 4.96, 150.86 ± 51.56 and >400 μg/ml for *Cladophoropsis* sp. The rest of the algal extracts were observed not to have significant cytotoxic effects in the concentration range from 6.25 μg/ml to 400 μg/ml. **Conclusion:** Our data conclusively suggest that *G. foliifera* and *Cladophoropsis* sp. may be good candidates for further fractionation to obtain novel anticancer substances. Moreover, stronger cytotoxic effects on estrogen negative breast cancer cell line (MDA-MB-231(ER^-)) in comparison to estrogen positive cells (MCF-7 and T-47D) suggest that the extract of *G. foliifera* and *Cladophoropsis* sp. may have an estrogen receptor/progesterone receptor-independent mechanism for their cellular growth inhibition.

Key words: Algae, Persian Gulf, Oman Sea, cytotoxicity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

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INTRODUCTION

Cancer is a leading cause of death worldwide. Studies show that in 2012, nearly 14.1 million new cases were diagnosed with cancer, and 8.2 million people died from the disease.[1] Among all types of cancer, breast cancer is the most common diagnosed cancer in women, ranking second after adding both genders together, and the leading cause of cancer-related death in women across the world.[1] In 2012, an estimated of 1.7 million women were diagnosed with breast cancer and almost 522,000 deaths occurred due to this disease.[1]

The most common breast cancer type is the invasive ductal carcinoma accounting for 70-80% of all breast cancers diagnosed.[2] Complement to breast mastectomy, radiation therapy and chemotherapy are frequently used for management of this malignancy. There are many chemotherapeutic agents used in the treatment of breast cancer. Nevertheless, due to the high side effects and resistance of cancer cells to these drugs,[3-5] there is still urgent need to develop new and more efficient therapeutic agents to battle the disease.

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Nature with its incomparable biodiversity of chemical agents has been the leading source for development of effective drugs. Nowadays, nearly 60% of all of the drugs used in cancer treatment are based on natural products.[6] Covering almost 70% of the earth's surface, the marine environment seems to be a treasury of novel bioactive compounds. Marine algae have been consumed as food in many parts of the world. They are rich in dietary fiber, minerals, polysaccharides, carbohydrates, lipids, proteins, and vitamins.[7,8] Recent studies have shown that seaweeds can be a source of new anticancer drugs.[9-16] Kahalalide F is an anticancer substance synthetized by the green algae, *Bryopsis* sp. Kahalalide F is now passing phase two of clinical trials for several solid tumors such as nonsmall cell lung cancer stage IIIB.[12,17] Various brown algae contain a sulfated polysaccharide named fucoidan in their fibrillar cell walls and intercellular spaces considered to protect the seaweeds against desiccation. Fucoidan has lately gone under various anticancer, cell cycle arrest, and apoptosis studies.[13,16] The results indicate that the substance has promising characteristics which may lead to a future anticancer marine drug.

The Southern parts of Iran have nearly 1,260 km coastline along the Persian Gulf and Oman Sea. The unique ecological properties of this area have led to the growth of some novel organisms in this region. 153 species of marine algae have been reported to live along the Bandar –e– Lengeh area at the south of Iran and north of the Persian Gulf.[18]

Only few studies have looked into the pharmacological properties of algae from Persian Gulf and Oman Sea.[19-25] The aim of this research was to determine the *in vitro* cytotoxic activity of total ethanol extracts (90%) of ten algae acquired from the coastlines of the Persian Gulf and Oman Sea against three human breast cancer cell lines; MDA-MB-231, MCF-7, and T-47D.

MATERIALS AND METHODS

Algae material

Algae samples were collected from the Iranian coasts of Persian Gulf and Oman Sea in 2008. The samples were collected by Dr. R. Rabii and Identified by Dr. J. Sohrabipour at the Agriculture and Natural Resource Research Center of Hormozgan, Iran. The voucher specimens of these algae were deposited at the Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. Information regarding the exact collection date and place of the algae samples and their voucher numbers are also demonstrated in Table 1. The seaweeds were washed twice with distillated water and stored at −20°C until use.

Table 1: Date and place of collection and voucher number of the algae samples

Algae	Collection date	Collection place	Voucher number
Botryocladia leptopoda	10-03-2008	Chabahar**	556
Cladophoropsis sp.	09-04-2008	Qeshm*	553
Colpomenia sinousa	05-03-2008	Bandar lengeh*	555
Cystoseira myrica	09-04-2008	Qeshm*	579
Gracilaria foliifera	09-04-2008	Bandar Abbas*	559
Gracilaria salicornia	09-04-2008	Qeshm*	554
Gracilariopsis longssima	08-04-2008	Bandar Abbas*	557
Hypnea flagelliformis	09-04-2008	Qeshm*	25Pm
Iyengaria stellate	10-03-2008	Bandar Moallem*	552
Laurencia papillosa	06-06-2008	Bushehr*	558

*Located in the Persian Gulf, **Located in Oman Sea

Preparation of algal extracts

Algal samples were freeze-dried and minced by a blender. 100 g of each alga was measured and macerated by ethanol (90:10) (2 × 1,000 ml). After filtration, the solvent was condensed and freeze-dried to obtain crude ethanol extracts. 20 mg of dried ethanol extracts was dissolved in 1 ml dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany) to prepare a 20 mg/ml stock solution of the extracts. The solution was passed through a 0.2 μm filter and kept in −26°C until use. For each experiment, the concentrations were prepared freshly.

Doxorubicin

Doxorubicin (Ebewe, Unterach, Austria), a current anticancer drug, was used as a positive control in our experiment. An available solution (2 mg/ml) of doxorubicin (Ebewe, Austria) was used as a stock. For each experiment, the working solutions were prepared freshly at 4°C and protected from light.

Cell lines

Three invasive breast ductal carcinoma cell lines, human MDA-MB-231 (estrogen receptor negative [ER^-]), MCF-7, and T-47D (both ER^+) were purchased from the National Cell Bank of Pasteur Institute of Iran. Cells were cultured in RPMI-1640 (Biosera, France) with 5% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Biosera, France) at 37°C in a humidified 5% CO_2 incubator. Cells were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 70% confluency.

3- (4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide assay

Cells were trypsinized with trypsin 0.25%, counted by trypan blue (Biosera, France) and suspended at a concentration of 3×10^4 cell/ml. 100 μl of this suspension was added to each well of a 96-well plate. Cells were left overnight to attach to the plate. After 24 h, cells

were inoculated, in triplicates, with quadruple-serially diluted concentrations of the algal extracts ranged from 6.25 μg/ml to 400 μg/ml. Negative controls for each extract concentrate were normal media containing the corresponding DMSO concentration. Doxorubicin was used at double-serially diluted concentrations between 0.9×10^{-2} μg/ml to 0.58 μg/ml. After 72 h of treatment, the medium was removed, and cell viability was measured by MTT assay. Briefly, 100 μl of MTT (0.5 mg/ml) in RPMI-1640 without phenol red was added to each well in the dark, and the plates were placed in the incubator for 4 h. Next, the supernatant was removed carefully, and 150 μl of DMSO (Merck, Germany) was added to each well. Plates were shacked for 15 min and were then read at 570 nm using a microplate reader. All the experiments were repeated three times. Cell cytotoxicity of each extract was calculated using the following equation:

$$\%\text{cytotoxicity} = 100 - (ABS_{test}/ABS_{control} \times 100\%).$$

Where ABS_{test} is the average absorbance of cells treated with algal extracts, $ABS_{control}$ is the average absorbance of corresponding DMSO control.

Statistical analysis

Statistical analysis was performed by Excel 2007 (Microsoft Corp., Redmond, Washington, USA) and inhibitory concentration (IC_{50}) values were determined using CurveExpert for Windows version 1.4 (Daniel Hyams, Hixson, USA).

RESULTS

The ethanol crude extract of ten algae from Persian Gulf and Oman Sea, as well as doxorubicin-as positive control were evaluated for their cytotoxic properties on three breast cancer cell lines, using MTT assay. Following 72 h of incubation, the IC_{50} values of doxorubicin on MDA-MB-231, MCF-7 and T-47D cell lines were 0.09 ± 0.03, 0.18 ± 0.04 and 0.37 ± 0.25 μg/ml, respectively.

The cytotoxic effect of the algae extracts is shown in Table 2. As illustrated in Table 2, *Gracilaria foliifera* and *Cladophoropsis* sp. extracts indicated the highest cytotoxic activity among the algae tested, and inhibited the cell growth in a dose-response manner. After 72 h of incubation, the IC_{50} values of *G. foliifera* on MDA-MB-231, MCF-7, and T-47D cell lines were 74.89 ± 21.71, 207.81 ± 12.07 and 203.25 ± 30.98 μg/ml, respectively. The IC_{50} values for *Cladophoropsis* sp. were 66.48 ± 4.96, 150.86 ± 51.56 and >400 μg/ml, respectively. The other algae extracts did not show any remarkable cytotoxic effect on the investigated cell lines in the concentration range from 6.25 μg/ml up to 400 μg/ml.

Table 2: Cytotoxic activity of the ethanol extracts of the algae after 72 h of incubation, assessed by MTT assay

Sample	IC_{50}* (μg/ml)±SD		
	MDA-MB-231	MCF-7	T-47D
Doxorubicin	0.09±0.03	0.18±0.04	0.37±0.25
Botryocladia leptopoda	>400	>400	>400
Cladophoropsis sp.	66.48±4.96	150.86±51.56	>400
Colpomenia sinousa	ND	>400	>400
Cystoseira myrica	ND	398±10.16	>400
Gracilaria foliifera	74.89±21.71	207.81±12.07	203.25±30.98
Gracilaria salicornia	ND	>400	>400
Gracilariopsis longssima	>400	>400	>400
Hypnea flagelliformis	>400	>400	>400
Iyengaria stellata	>400	>400	>400
Laurencia papillosa	ND	>400	>400

*50% inhibitory concentration; ND=Not detected; SD=Standard deviation; MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

DISCUSSION

Marine seaweeds have been considered as a source of new anticancer drugs.[12,15,16] In the present study, *in vitro* cytotoxic activity of the total ethanol extracts (90%) of ten algae acquired from the coastlines of the Persian Gulf and Oman Sea was determined against three human breast cancer cell lines; MDA-MB-231, MCF-7, and T-47D. Results indicated *G. foliifera* and *Cladophoropsis* sp. to have the highest cytotoxic effect on the investigated cell lines.

The ethanol extract of the red alga *G. foliifera* showed the IC_{50} values of 74.89 ± 21.71, 207.81 ± 12.07 and 203.25 ± 30.98 μg/ml against MDA-MB-231, MCF-7, and T-47D cells, respectively. Many *Gracilaria* species are frequently used as food in various countries and are also of great interest for commercially producing food grade agar.[26,27] To our knowledge, no study to date, has investigated the cytotoxic effect of *G. foliifera* species. However, other species of this genus have undergone investigations in different studies. Two compounds found in *G. asiatica* (gracilarioside and gracilamides) have shown mild cytotoxic effects against A375-S2 melanoma cell line.[28] Evaluating the effects of *G. salicornia* extract from the Persian Gulf and *G. corticata* extract from India on brine shrimp, in different studies, revealed the potent cytotoxic activity of these algae with the LC_{50} of 3 μg/ml[29] and 1.081 μg/ml,[30] respectively. *G. corticata* from Persian Gulf was also reported to have potent cytotoxic effects on Jurkat and MOLT-4,[19] MCF-7, MDA-MB-231, HeLa, HepG2 and HT-29[24] human cancer cell lines. Sundaram *et al.* have

shown an increase in the life span and an inhibition of tumor formation in Ehrlich ascites carcinoma bearing mice which were treated by ethanol extract of *G. edulis*.[31] It is notable that in our study the ethanol extract of *G. foliifera* was more active than *G. salicornia* against the breast cancer cell lines [Table 2]. These data, collectively with our observation, suggest that the members of *Gracilaria* genus, including *G. foliifera* merit more investigations as the anticancer candidates.

As shown in Table 2, *Cladophoropsis* sp. ethanol extract had the IC_{50} values equal to 66.48 ± 4.96, 150.86 ± 51.56 and >400 μg/ml against MDA-MB-231, MCF-7, and T-47D cells, respectively. So far, there have only been several reports dealing with the cytotoxic effects of this algal genus,[32-34] and our study is the first to investigate the cytotoxic effects of this alga on breast cancer cell lines. In a research by Kanegawa *et al. C. zollingeri* extract was observed to have moderate telomerase inhibitory effect on MOLT-4 leukemic cell line.[34] Studies done by Harada *et al*. on 306 marine algae revealed that the methanol extract of *C. vaucheriaeformis* with the concentrations of 50–100 μg/ml has a high cytocidal effect on L1210 murine leukemic cells.[32] The cytotoxic effect of *C. vaucheriaeformis* was further evaluated on five human leukemia cell lines. The results indicated that the algal extract have much weaker effects on human leukemic cell lines than murine derived cell lines. *C. vaucheriaeformis* extract had also possessed a selective cytotoxicity on murine malignant cells[32] but unluckily no conspicuous selective cytotoxic activity was detected on human malignant cells.[33] In our study, *Cladophoropsis* sp. exhibited an 80% cell growth inhibition on MDA-MB-231 cells at the concentration of 100 μg/ml. Although we did not have normal human cell lines in our experiment; the effect of *Cladophoropsis* sp. is less but close to the effect of *C. vaucheriaeformis* on human leukemia cell lines; HL60 and MOLT-4. Together our observation, along with the data from other investigations suggest that the members of the alga genus *Cladophoropsis* might contain valuable cytotoxic metabolites against cancer cells.

The results of this study also showed that the cytotoxic effect of the algae *G. foliifera* and *Cladophoropsis* sp. on the ER/PR/HER2 triple negative breast cancer cell line (MDA-MB231) is stronger than that of estrogen-receptor positive cell lines (MCF7 and T47D) ($ER^+/PR^+/HER2^-$). These data suggest that the growth inhibition properties of these two algae extract might be independent from cell ERs.[35,36] As triple negative tumors are believed to be more invasive with higher reoccurrence rate that ER/progesterone receptor positive tumors,[37] this observation may shed light on the new aspects of anticancer agents. The observation; however, needs to be evaluated in other investigations in which proteome profiling of the target cells is assessed.

Although we did not demonstrate significant cytotoxic effects of the other algae, genera *Colpomenia*,[38,39] *Cytoseira*,[36,40-43] *Gracilariopsis*,[44] *Iyengaria,*[45] and *Laurencia*,[46] from other regions of the world, have exhibited cytotoxic effects in other studies.

Totally, our data indicated that among ten algae ethanol extracts from Persian gulf and Oman Sea listed in Table 2, *G. foliifera* and *Cladophoropsis* sp. might be considered for further fractionation and *in vitro*, as well as *in vivo*, anti-cancer investigations.

A limitation of our study should, however, not to be ignored. Evaluating the effects of the extracts on normal human cell line may reveal the selectivity of the investigated extracts.

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