Cytotoxicity of fucosterol containing fraction of marine algae against breast and colon carcinoma cell line

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ABSTRACT

Context: Marine algae produce different secondary metabolites with a wide range of biological activities. Many studies have been achieved on the screening of biological effects of marine organisms and a lot of active compounds were isolated and characterized. **Aims:** In an attempt to find cytotoxic compound of hexane fraction, isolation, identification, and cytotoxicity of active compound of this fraction were performed. **Materials and Methods:** In this study, total methanolic (70%) extract and partition fractions of hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and MeOH–H_2O of *Sargassum angustifolium*, *Chondria dasyphylla*, and *Ulva flexuosa*, collected from coastlines of the Persian Gulf in south of Iran, were studied against colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), breast ductal carcinoma (T47D), and Swiss mouse embryo fibroblast (NIH 3T3) cell lines by MTT assay. **Statistical Analysis Used:** IC_{50} (median growth inhibitory concentration) values were calculated by Sigmaplot (10) software. **Results:** Hexane fraction of *Chondria dasyphylla* (IC_{50} 82.26 ± 4.09 μg/ml) and MeOH-H_2O fraction of *Ulva flexuosa* (IC_{50} 116.92 ± 8.58 μg/ml) showed cytotoxic activity against proliferation of T47D cells. Hexane fraction of *Sargassum angustifolium* was also observed for cytotoxicity against T47D and HT-29 cell lines (IC_{50} 166.42 ± 26.7 and 190.24 ± 52.8 μg/ml), respectively. An investigation of a component from the hexane fraction of *Sargassum angustifolium* yielded a steroidal metabolite, fucosterol, with cytotoxicity in T47D and HT29 (IC_{50} 27.94 ± 9.3 and 70.41 ± 7.5 μg/ml). **Conclusions:** These results indicated that fucosterol, the most abundant phytosterol in brown algae, is responsible for cytotoxic effect of this extract against breast and colon carcinoma cell lines.

Key words: Cytotoxic activity, fucosterol, marine algae, Persian Gulf

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INTRODUCTION

For many years, man has used the sea as the producing resource of useful economical materials and supplements. Seaweeds (macroalgae), mainly brown algae, are used as sea vegetables in the food basket, especially in the sea adjacent countries and Far East ones such as China, Japan, and Korea. In the south of Iran, people use seaweeds in their daily foods. Phytochemical investigations on plants which have been used in traditional medicine for cancer treatment, has led to separation of compounds with antitumor properties. Cytotoxic antitumor compounds isolated from marine organisms have been reported in several sources during the 40 years.[1] Halomon isolated from a red alga[2] is under clinical trial phases and will be offered as pharmaceutical products in the future. Secondary metabolites with antitumor activity have been extracted and identified in *Sargassum thunbergii*, *Sargassum horneri*,[3] *Sargassum tortile*[4] and *Sargassum crispom*.[5] Also antiviral and cytotoxic steroids of *Sargassum carpophyllum*[6] have been reported. Studies on *Ulva lactoca*, as a diet full of oligosaccharide, showed cytotoxic effect and it also can be a choice for epithelial cancer and normal cells.[7] Red algae from Chondria are known as producers of cyclopolysolfieds, terpenoides, novel amino acids, and amines. *Chondria armata*, a red alga as a source of domoic acid derivatives, has larvicidal and lowering blood pressure effects.[8]

The Iranian coastlines in the Persian Gulf and Oman

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Sea are about 1260 km long. Recent data have shown 153 species of marine algae from coastlines of Iranian islands and Hormozgan province.[9,10] However, only a few studies have been done about pharmacological effects of the marine algae of this region. This study was designed to determine *in vitro* cytotoxic activity of MeOH (70%) extract and partition fractions obtained from three species of the algae, brown alga *Sargassum angustifolium*, red alga *Chondria dasyphylla* and green alga *Ulva flexuosa* from coastlines of the Persian Gulf in southern Iran. In addition, isolation, identification, and cytotoxicity of a compound from cytotoxic hexane fraction of *S. angustifolium* were performed.

MATERIALS AND METHODS

Plant material

Brown alga, *Sargassum angustifolium* C. Agardh (Sargassaceae), red alga, *Chondria dasyphylla* (Woodward) C. Agardh (Rodomelacea), green alga, *Ulva flexuosa* Wulfen (Ulvaceae), were collected in February 2008 from Asaluye-Niband marine protected area of the Persian Gulf. The algae were identified by Dr. J. Sohrabipour at the Agriculture and Natural Resource Research Center of Hormozgan, Iran. The voucher specimens were deposited in this center.

Extraction of marine algae

The algae were air –dried in the shade at room temperature and ground to powder with a mortar and pestle. Fifty grams of each sample were extracted with MeOH-H_2O (70%) (5×200ml) at room temperature. The combined extracts were evaporated under vacuum. The residues were successively partitioned between MeOH–H_2O (9:1) and Hexane, MeOH-H_2O (8:2) and $CHCl_3$, MeOH-H_2O (1:1) and EtOAc. Removal of the solvents resulted in the productions of Hexane, $CHCl_3$, EtOAc and MeOH-H_2O fractions.

Cell culture

Colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), and breast ductal carcinoma (T47D) cell lines were maintained as exponentially growing cultures in RPMI 1640 cell culture medium (PAA, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) for HT-29 cells and 15% FBS for Caco-2 and T47D cells. The **S**wiss mouse embryo fibroblast (NIH 3T3) cell line was kept in Dulbecco's Modified Eagle's Medium (DMEM; PAA, Germany) supplemented with 10% FBS. 100 IU/ml penicillin and 100 µg/ml streptomycin (Roche, Germany) were added to the media. All cell lines were cultured at 37 °C in air/carbon dioxide (95:5) atmosphere.

Determination of cell viability by mitochondrial tetrazolium test assay

The concentration of 5, 50, 150, 450, and 900 µg/ml from all samples including total methanolic extract and partition fractions were tested for each cell line. In an extension of our study, the concentration of 4.5, 18, 36, and 72 µg/ml of fucosterol, isolated from hexane fractions of *Sargassum angustifolium* and *Chondria dasyphylla*, were examined against each cell line. Samples were dissolved in DMSO (dimethyl sulfoxide) and further diluted with cell culture medium. The final DMSO concentration used was 1% of total volume of medium in all treatments, including the control group. Cells with no treatment and methanolic (80%) extract of *Vinca rosea* treatment were examined as negative and positive control, respectively.

For mitochondrial tetrazolium test (MTT) assay, 1×10^4 cells /well were plated into 96-well plates (Nunc, Denmark) and incubated for 24 h before addition of extracts. The plates were allowed to proliferate and reach their exponential phase of growth. The incubation time for each cell line was assigned according to the normal growth curve of that cell line and was determined twice as long as the doubling time of each cell line. After 72 h of incubation for HT-29 cells, 96 h for T47D and NIH 3T3 cells, and 120 h for Caco-2 cells, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Merck, Germany) reagent (5 mg/ml) in phosphate buffered serum (PBS) was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the medium was removed and 100 µl cell culture grade DMSO was added to each well. The formazan salts were quantified by reading the absorbance at 550 nm on a microplate reader (Anthos, Austria).[11]

Cell viability in MTT assays was calculated as a percentage of untreated cells (control value). The cytotoxicity value was presented as IC_{50} (the median growth inhibitory concentration) of the reagents compared to control.

Isolation of fucosterol

Hexane fraction of *Sargassum angustifolium* was chromatographed on preparative thin-layer chromatography (PTLC) by using silicagel plates (20 cm × 20 cm) and chloroform-ethyl acetate (7:3) to afford the compound **1**.

Chemical analysis

^1H and ^{13}C NMR spectrum was recorded on a Brucker Avance 500 DRX (500 MHz) spectrometer. Chemical shifts are given in (ppm) $CDCl_3$ using TMS as internal standard. EI-MS spectra were recorded on Agilent Technology (HP) instrument.

Compound **1**; EI-MS *m/z*: 412 $[M]^+$ (18), 397 (7), 379 (8), 314 (100), 299 (25), 296 (26), 281 (34), 271 (18), 255 (11), 253 (6), 229 (25), 213 (26); m.p. (°C): 124 °C, ^1H NMR (500 MH_z, $CDCl_3$) δ_H (ppm): 5.35 (1H, br d, H-6), 5.18 (1H, q,

H-28), 3.52 (1H, m, H-3), 2.2 (1H, sep, H-25), 1.57 (3H, d, H-29), 1.01 (3H, s, H-19), 0.99 (3H, d, H-21), 0.98 (3H, d, H-27), 0.97 (3H, s, H-26), 0.69 (3H, s, H-18); ^{13}C NMR (125 MH_z, $CDCl_3$) δ_C (ppm): 147.01 (C-24), 140.78 (C-5), 121.70 (C-6), 115.5 (C-28), 71.82 (C-3), 56.78 (C-14), 55.82 (C-17), 50.1 (C-9), 42.33 (C-13), 39.78 (C-12), 37.28 (C-1), 36.53 (C-10), 36.43 (C-20), 35.2 (C-22), 34.78 (C-25), 31.93 (C-7), 31.9 (C-8), 31.69 (C-2), 28.24 (C-16), 25.73 (C-23), 24.33 (C-15), 22.24 (C-27), 22.13 (C-26), 21.10 (C-11), 19.40 (C-19), 18.7 (C-21), 13.17 (C-29), 11.58 (C-18).

RESULTS

Cytotoxicity assay

Among all methanolic extract and fractions of *Sargassum angustifolium*, chloroform and ethyl acetate fraction showed cytotoxicity in all studied cell lines. Hexane fraction of this alga indicated cytotoxic activity against T47D and HT-29 cell lines (IC_{50} 166.42 ± 26.7 and 190.24 ± 52.8 μg/ml) and showed low cytotoxic effect against NIH3T3 cell line [Table 1].

Chloroform fraction of *Chondria dasyphylla* exhibited potent cytotoxicity against T47D cell line (IC_{50} 33.54 ± 4.60 μg/ml) and hexane fraction of this sample also showed cytotoxic effect against T47D cell line (IC_{50} 82.26 ± 4.09 μg/ml) [Table 1]. MeOH-H_2O fraction of *Ulva flexuosa* showed cytotoxic effect against T47D (IC_{50} 116.92 ± 8.58 μg/ml) and chloroform fraction of this alga exhibited cytotoxicity against NIH3T3 and T47D (IC_{50} 60.90 ± 9.9 and 80.28 ± 7.35 μg/ml) [Table 1]. Hexane fraction (250 mg) from methanolic extract (70%) of the brown alga *Sargassum angustifolium* was chromatographed on silicagel PTLC using chloroform:ethyl acetate (7:3) as solvent to isolate compound 1 (5 mg). Compound **1**, a white solid, showed its molecular ion at *m/z* 412 in its EI-MS spectrum. ^{1}H NMR and ^{13}C NMR data of compound 1 exhibited the typical pattern for fucosterol [Figure 1].

Fucosterol showed cytotoxicity against T47D and HT29 cell lines (IC_{50} 27.94 ± 9.3 and 70.41 ± 7.5 μg/ml), respectively. This metabolite exhibited none toxic effect in Caco-2 and NIH 3T3 up to 70 μg/ml.

DISCUSSION

The cytotoxicity of natural products is based on the presence of antitumor metabolites. More than 140 secondary metabolites from Phaeophyceae have been reported.[12] This study on total extract and different fractions of *Sargassum angustifolium* represented cytotoxicity of chloroform and ethyl acetate fractions of this alga against all studied cell lines while hexane fraction of this plant showed good cytotoxicity in T47D and HT-29 cell lines without cytotoxic effects on the normal cell line (NIH3T3). Cytotoxic activity of this alga could be related

Table 1: IC_{50} values (μg/ml) of methanolic extract and partition fractions of three species of algae and methanolic extract of *Vinca rosea* (as positive control) against tumor and normal cell lines. Results are expressed as mean ± SD

Sample	Cell lines (MTT assay)[a]			
	T47D	Caco-2	HT-29	NIH 3T3
S. angustifolium				
Total extract	>900	>900	>900	>900
MeOH-H_2O fr.	>900	>900	>900	>900
Hexane fr.	166.42 ± 26.7	317.11 ± 42.15	190.24 ± 52.8	735.48 ± 71.90
Chloroform fr.	86.99 ± 4.60	186.68 ± 10.11	299.68 ± 120.9	162.69 ± 305.8
Ethyl acetate fr.	117.88 ± 10.74	101.29 ± 9.62	261.63 ± 137.99	165.37 ± 30.3
C. dasyphylla				
Total extract	> 900	> 900	> 900	> 900
MeOH-H_2O fr.	522.56±72.63	>900	>900	> 900
Hexane fr.	82.26 ± 4.09	421.11 ± 134.0	>900	>900
Chloroform fr.	33.54 ± 4.66	144.61	114.51 ± 2.1	112.37 ± 9.6
Ethyl acetate fr.	167.26 ± 27.3	490.60 ± 89.6	595.94 ± 114.83	505.09 ± 99.33
U. flexuosa				
Total extract	554.5 ± 8	>900	> 900	> 900
MeOH--H_2O fr.	116.92 ± 8.58	> 900	> 900	> 900
Hexane fr.	> 900	107.64 ± 2.38	291.25 ± 365	194.66 ± 9.5
Chloroform fr.	80.28 ± 7.35	189.69 ± 16.20	183.87 ± 15.17	60.90 ± 9.9
Ethyl acetate fr.	> 900	175.44 ± 198.18	364.22 ± 19.26	212.56 ± 41.03
Vinca rosea	195.78 ± 17.96	571.32 ± 5	412.09 ± 43.55	>900

[a]Key to cell lines employed: HT-29 and Caco-2 (colon adenocarcinoma), T47D (breast carcinoma), NIH 3T3 (Swiss embryo fibroblast)

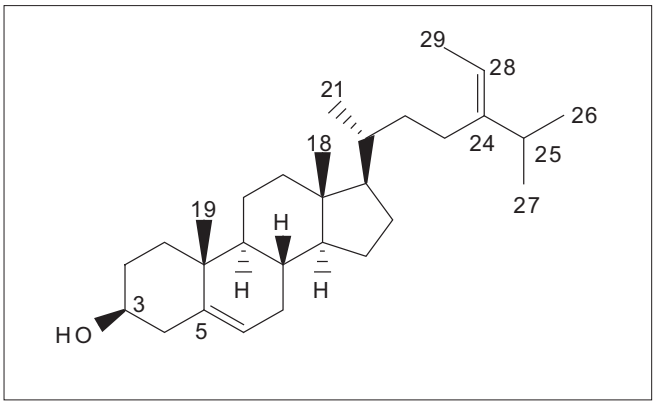

Figure 1: Structure of fucosterol: a cytotoxic compound isolated from hexane fraction of methanolic extract of *Sargassum angustifolium*

to presence of diterpenes, phlorotannins,[12] and sulfated polysaccharides.[13,14]

Red algae contain mainly isoprenoid and acetogenines derivatives, along with some amino acids, shikimate, and nucleic acid derivatives. They also are significant producers of halogenated compounds with more than 90% of bromine or chlorine,[15] cholesterol, and desmosterol in marine environment. Cyclic polysolfides, terpenoides, amino acids, and indolic metabolites have been found in *Chondria* species.[16,17] According to the results of this study, hexane fraction of *Chondria dasyphylla* could contain phytosterols with cytotoxicity against breast cancer cell line. Chloroform fraction of this sample showed the most cytotoxic effect against T47D cell line and in the rest of the cell lines including normal cell line, cytotoxicity was observed. Above-mentioned compounds can be responsible for these effects.

Among all macro algae, green algae with less than 300 known compounds are the least producers of natural compounds.[15] The study done by Celikler (2009) indicated that green alga *Ulva rigida* show antimutagenic effect related to the antioxidant compounds in the ethanol extract. It also indicated a strong antioxidant and antidiabetic effects.[18] Different extracts of *Ulva pertusa*, which has been considered as an antitumor Chinese medicine, showed cytotoxic activity to both normal and tumor cells.[19] In current research, total extract and MeOH-H_2O fraction of *Ulva flexuosa* showed cytotoxic activity against T47D cell line; this effect was higher in MeOH-H_2O fraction than total extract. The results of this study in comparison with the cytotoxicity of total extract of *Vinca rosea,* which contains anticancer compounds, vincristine and vinblastine, showed that total extract and MeOH-H_2O fraction of *Ulva flexuosa* can be regarded for future studies. Chloroform fraction of this alga showed stronger cytotoxic effect against normal cell line than the cancer cell lines.

Based on the results of present study we identified fucosterol from the hexane fractions of methanolic extract of brown alga *Sargassum angustifolium* as a cytotoxic compound. Fucosterol, the most abundant sterol in brown algae,[20] has been shown to be antioxidant,[21] antidiabetic,[22] antifungal,[23] and cytotoxic.[6] In our previous study,[24] hexane fractions of *Sargassum swartzii* and *Cystoseira myrica* showed cytotoxicity against Caco-2 and T47D cell lines, respectively and they were observed for increasing apoptosis in Caco-2 and T47D cells. Presence of fucosterol in these fractions and also in hexane fraction of *Chondria dasyphylla* was confirmed by TLC.

Phytosterols have been founded as cancer preventive substances. Several studies have indicated that phytosterols are toxic to breast, colon, and prostate cancer cells. There are evidences that some phytosterols have antioxidant activity.[25] Phytosterol especially beta-sitosterol was shown not only to inhibit growth, but also to induce apoptosis in human breast and stomach cancer cells. Phytosterol supplementation may alter or delay tumor progression.[26,27]

CONCLUSION

This study indicates that fucosterol present in marine algae can be used as a prototype for development of new drugs and/or as a source of antitumor pharmaceutical raw material. Further investigations may include the assessment of underlying mechanisms of action of this compound and evaluation of its *in vivo* efficacy in animal models, which could not be performed in the current study due to low available amounts of the compounds.

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