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RESEARCH ARTICLE

Identification and anti-cancer activity in 2D and 3D cell culture evaluation of an Iranian isolated marine microalgae *Picochlorum* sp. RCC486

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Abstract

Purpose Cancer disease is the second cause of death in the world. Now a days, high percentage of drugs, which are involved in treatment of cancers, have natural origin. Introduction of microalgae strains as anti-cancer drugs origin is a valuable approach for cancer therapy.

Methods In the present study we describe the isolation, characterization, and anti-proliferative activity of a new microalga strain (*Picochlorum* sp. RCC486) from Iran. The cytotoxic activity of four different algal extracts including methanol, ethyl acetate, chloroform, and hexane were evaluated against MDA-MB-231, MCF-7, Hep-G2, and A-549 cell liens. Cell viability was determined using MTT assay in both monolayer and spheroids 3D cultures. The apoptosis was confirmed by different methods such as AO/EB and Annexin V-FITC/PI double staining, caspase-3 colorimetric assay, ROS and MMP assay.

Results The results of MTT assay and fluorescent double staining confirmed that methanol and ethyl acetate extracts showed the best cytotoxic activity against the cancer cell lines. The production of ROS, caspase-3 activity and depolarized MMP were quite significant in MDA-MB-231 cell line treated with methanol and ethyl acetate extracts.

Conclusion In this research we revealed that cytotoxicity and apoptotic effects of the methanol and ethyl acetate extracts in human cancer cells make them good candidates for further pharmacological studies to discover effective drugs for cancer therapy.

Keywords Picochlorum · Microalgae · Cancer · Apoptosis

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Introduction

The cancer incidences are increasing and will likely to continue for decades. Cancer treatments, as a dreadful human disease, do not have effective remedy as the currently available drugs are causing negative adverse effects [1]. Apoptosis is a physiological process of cellular suicide which plays an important role in the pathogenesis of cancer. Reduction in apoptosis or apoptosis resistance plays a vital role in carcinogenesis [2, 3]. Disruption in the balance of pro-apoptotic and anti-apoptotic members of the Bcl-2 family, decreased expression of caspases and impaired death receptor signaling cause dysregulated apoptosis in the affected cells [4]. It has been recognized that evasion of apoptosis facilitate cancer development by promoting angiogenesis, blocking differentiation and increasing cell motility, invasion, and metastasis [5]. Many cancer therapeutical drugs exert their effects through reactive oxygen species (ROS) generation [6]. Low levels of ROS function as

signaling molecules to regulate a wide variety of physiological processes, while excess ROS due to disturbance in the cellular redox homeostasis promote cell death [7].

Stimulation of extrinsic death receptor apoptotic pathway by ROS induce receptor clustering and formation of lipid raft-derived signaling platforms. ROS disturb mitochondrial membrane potential and leading to the release of mitochondrial apoptogenic factors like cytochrome c, AIF, and Smac/Diablo that trigger caspase-dependent or caspase-independent cytosolic signaling events [8]. The anti-cancer treatment approaches could be basis on molecules that prevent the ROS formation precluding carcinogenesis or relies on chemical agents that cause a sudden increase of ROS leading to significant stress oxidative inside tumor mass [9].

There has been an intense effort over the past decade to find new anti-cancer lead compounds from diverse marine life. An assessment of documents reveals natural products and their derivatives represent over half of all the drugs in clinical use up to now. Almost 60% of FDA approved drugs for the treatment of cancers are originally derived from natural sources [5]. Since historically, marine environment has proven to be a rich source of potent natural compounds such as peptides, steroids, macrolides, polyketides, alkaloids, terpenes, polyphenolics and so forth. Interestingly, some marine agents are proved to be the potent sources of drugs. Spongothymidine, as lead compound for the discovery of cytarabine, was one of the first marine-derived anti-cancer agents. It is anticipated that the marine microalgae will become an worth source of novel compounds in the future, because of pharmacological properties of algal metabolites for disease prevention and treatment [10, 11]. Studies on the bioactivities of marine algae revealed that chemopreventive agents prevent growth of cancer cells and induce apoptosis by targeting signaling molecules, such as caspases [12]. The cytotoxicity mechanism of some components of microalgae extracts have been proved that could be mentioned to generation of ROS, cleavage of caspases-3 and -9 and poly-ADP-ribose polymerase (PARP), changes in Bcl-2 family levels and DNA fragmentation [13].

In this research, microalgae strain collected from the Persian Gulf coast has been isolated and identified as *Picochlorum* sp. RCC486. The cytotoxicity of the methanol, ethyl acetate, chloroform and hexane fractions against human breast, lung and liver cancer cell lines was evaluated. Since apoptosis is described as the main mode of cell death induced by chemotherapies in cancer cells, so we evaluated the ability of these extracts to induce apoptosis in human cancer cell lines.

Methods

Strain and culture condition

The microalgae used in this study (*Picochlorum* sp. RCC486) was isolated from Persian Gulf (26°32 N, 53°56 E) at the southern of Iran and deposited in Persian Type Culture Collection (PTCC) as NO. 6032. Water samples were collected and plated on petri dishes with Bold's Basal Medium (BBM) and 1% agar. The culture medium is included 250 mg $NaNO_3$, 100 mg K_2HPO_4, 150 mg KH_2PO_4, 75 mg $MgSO_4$, 25 mg $CaCl_2$, 25 mg NaCl, 1.44 mg $MnCl_2$, 0.71 mg Na_2MoO4, 11.4 mg H_3BO_3, 8.82 mg $ZnSO_4$, 0.08 mg $FeSO_4$, 1.57 mg $CuSO_4$, 0.49 mg $Co(NO_3)_2$, and 50 mg EDTA per 1 L distilled water.

After sequential subculturing, single colonies were picked and re-suspended into a new medium. In order to inhibit the growth of possible contaminant bacteria antibiotics were added to isolated colonies.

Microalgal strain was pre-cultured in 250 mL erlenmeyer flasks with 150 mL of culture media by shaking at 110 rpm and 25 °C that was illuminated by cool white fluorescent lamps at an intensity of 2700 lx in 16:8 h light-dark cycles. After 15 days the inoculum concentration was about 5.5×10^7 cells/mL. The biomass was harvested by centrifuging at 1500×g for 20 min at the end of the logarithmic phase and the wet biomass was freeze-dried and stored in refrigerator at 4 °C. The cell density was measured daily by a spectrophotometer at 620 nm. The biomass productivity was calculated by optical density (OD) of the cells. Biomass concentration, on the other hand, was determined by dry weight (DW) measurements which is conducted by filtering of 10 mL of the cell suspension through filter paper (Whatman GF/F) and washing the filters with distilled water. Ultimately, the filters were oven-dried at 80 °C for 24 h and cooled in a desiccator and weighted. Dry weight was calculated from the difference between initial and final weight. The number of cells were obtained by counting in a neubauer chamber using an optical microscope. All experiments were conducted three times independently.

Genomic DNA isolation

The biomass was harvested by centrifugation and the resulting pellet added into a 1.5 mL eppendorf tube with 500 μL of lysis buffer (Tris-HCl, pH 8.0, 400 mM, EDTA, pH 8.0, 60 mM, NaCl 150 mM, sodium dodecyl sulfate 1%) and incubated at room temperature for 10 min. The 150 μL of potassium acetate (pH 4.8) was added into the solution and the mixture vortexed during 15 min and spun at 10,000×g for 1 min. The supernatant centrifuged again as described above and transferred to a new tube and equal volume of isopropyl alcohol was added into solution and mixed by inversion briefly.

Ultimately the tube was centrifuged at 10,000×g for 2 min after removing the supernatant the resultant DNA pellet was washed in 300 μL of 70% ethanol and spun at 10,000×g for 1 min. The supernatant is discarded again and the DNA pellet dried, and re-suspended in 50 μL of 1xTris-EDTA, pH 8 buffer. The amount of purified DNA is1 μL for 25 to 50 μL of PCR mixture. Quantification of the genomic total DNA obtained and assessment of its purity was done on a nanodrop Epoch™ Microplate Spectrophotometer (BioTek).

Amplification of 18S rRNA encoding gene

Amplification of microalgal 18S rRNA gene fragments was carried out by PCR. The total volume of the reaction was 25 μL. 1 μL of genomic DNA, 10 pM of SSU850 (F) (GGGACAGTTGGGGGTATTCGTA) and 10 pM of SSU870 (R) (TACGAATACCCCCAACTGTCCC) universal primers, 0.2 mM dNTPs, 0.5 U Taq DNA polymerase from CinnaGen (Tehran, Iran) 2.5 μL of specific 10xbuffer (containing 2.5 mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO) were added into a Eppendorff thermo-cycler and the PCR program (96 °C for 30 s, 58 °C for 30 s, and 72 °C for 120 s) was performed for 30 cycles.

Preparation of the extracts

In order to preparation of the extracts 200 mg of ground freeze dried microalgae was extracted for 24 h with 2 mL of different polarity solvents hexane, chloroform, ethyl acetate and methanol at room temperature. The tube was centrifuged at 1500×g for 10 min and the supernatant was recovered and filtered. The residue was subsequently extracted three times, and the supernatants of each solvents were mixed.

Total phenolic determination

Extracts phenolic content was determined by the Folin–Ciocalteu method. For measuring the phenolic concentration of each sample, 20 μL different concentrations of extract (50, 100, 150, 250, 500 mg/L final concentrations) mixed with 1.58 mL distilled water, 100 μL Folin–Ciocalteu reagent and 300 μL sodium carbonate and allowed to stand for further 60 min in the dark at room temperature and absorbance was measured at 765 nm.

Carotenoid determination

Carotenoid content was determined spectrophotometrically based on Lichtenthaler and Buschmann method and the content was calculated by Lichtenthaler equations [14]. Using 90% (*v*/v) methanol in water, aliquots of the extracts were diluted 15–300 times and the absorbance was determined at 470, 652 and 665 nm.

Cell lines and cell culture

Four different human cancer cell lines including MDA-MB-231, MCF-7 (human breast cancer cell lines), Hep-G2 (human liver cancer), A549 (human lung cancer) and HDF (Human Dermal Fibroblasts) were purchased from National Cell Bank of Iran (NCBI). The cells were cultured in RPMI 1640 (GibcoeBRL, UK) medium supplemented with 10% heat-inactivated fetal calf serum (GibcoeBRL, UK) and 100 mg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere with 5% CO_2.

In vitro cytotoxicity assay

Cell viability was determined by MTT [3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay in triplicate. The cytotoxicity of hexane, chloroform, ethyl acetate and methanolic extracts of microalgal strain with different final concentrations (100 μg/mL, 200 μg/mL, 400 μg/mL and 500 μg/mL) were tested against four human cancer cell lines and one normal cell line. The reference drug, etoposide, was used with final concentrations as 1, 5, 10 and 20 μg/mL as positive control. The cells were harvested by trypsinization and diluted to 1×10^4 cells/mL. 190 μL of the cell suspension from different cell lines was seeded in 96-well culture plate and incubated overnight in a humidified air atmosphere at 37 °C with 5% CO_2. The cells were treated with 10 μL of various concentrations of the extracts and solvent DMSO (1%) as negative control and incubated for further 48 h. At the end of incubation the medium was removed and 200 μL phenol red-free medium containing MTT (1 mg/mL) was added to each well and incubated for further 4 h. After incubation, supernatants were discarded, and 100 μL of DMSO was added to each well. The absorbance values were determined with multi-well plate reader (Gen5, Epoch, BioTek, America) at 492 nm. The inhibition percent and IC_{50} values compared with the negative control were calculated by nonlinear regression analysis and expressed in Mean ± SD. The experiments were conducted three times.

Acridine orange/ethidium bromide (AO/EB) double staining method

Morphological changes in treated cells were evaluated by fluorescence microscopy using acridine orange/ ethidium bromide double staining (AO/EB). The cell suspension (3×10^5 cells/well) of cancer cells (MDA-MB-231, MCF-7 and Hep-G2) were cultured in 6-well plates and incubated overnight. Then, cells were treated with IC_{50} concentration of most potent cytotoxic extracts and etoposide and incubated for 24 h. After harvesting and washing with phosphate buffer saline (PBS), cells were stained with dye mixture (1:1, 100 μg/mL) acridine orange and ethidium bromide. Stained cells

suspension (10 μL) were placed on a clean microscope slide and covered with a coverslip and examined immediatly by fluorescence microscope (Axoscope 2 plus, Zeiss, Germany).

Flow cytometry analysis of the apoptotic cells with FITC annexin V and PI double staining

The FITC annexin V/PI apoptosis detection kit (BD Pharmingen™) was used to detect and quantify apoptosis by flow cytometry as per manufacturer's instructions. In brief, 3×10^5 cells/well of Hep-G2 and MDA-MB-231 cells were treated with IC_{50} dose of most potent extracts and etoposide. After 24 h incubation, the cells were harvested and washed twice with cold PBS. Then the cell suspension was centrifuged, and re-suspended in 1× annexin V binding buffer and were double stained by adding 5 μL of FITC annexin V and 5 μL of PI. The cells were gently vortex and incubated at room temperature for 15 min. After incubation, 400 μL of 1× annexin binding buffer was added into the suspension and cells were analyzed using a flow cytometer with in 1 h.

Caspase-3 activation assay

The caspase-3 activity was measured using caspase-3 colorimetric assay. MDA-MB-231 and Hep-G2 cells were seeded in 6-well plate at the density of 3×10^5 cells/well and treated with IC_{50} concentrations of most potent extracts and etoposide for 24 h. After incubation the cells were harvested and washed with PBS. Then cell pellets were re-suspended in ice-cold cell lysis buffer (20 mM PIPES, 10 mM KCl, 2 mM $MgCl_2$, 4 mM DTT, 2 mM EDTA and 1 mM EGTA freshly supplemented with protease inhibitors) for 10 min. At the end of the incubation, cell lysates were centrifuged for 15 min (10,000×g) and then the cytosol fraction was transferred to fresh tubes and put on ice for performing the assays. One hundred micrograms protein was used for each assay in the presence of 200 μM colorimetric caspase substrate (Ac-DEVD-*p*NA, Sigma) in 100 μL reaction buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 0.1% CHAPS, 0.1 mM EDTA, 10% Glycerol). Protein concentrations were measured by the Bradford method and bovine serum albumin was used as the standard. The OD was determined at 405 nm (every 60 s for 120 min) by an ELISA reader. Caspase-3 activity was expressed as the change of the activity in treated cancer cells compared to the untreated controls.

Reactive oxygen species (ROS) assay

ROS production was measured using 2′, 7′-dichlorodihydrofluorescin (DCFH-DA). MDA-MB-231 and HDF cells were cultured in 6-well plate at a density of 3×10^5 cells/well and treated with IC_{50} concentration of ethyl acetate, methanol extracts and etoposide for 24 h. After incubation time, treated cells were collected and centrifuged at 200×g for 5 min. Ultimately, the pellet was washed with PBS and the samples were stained with DCFH2-DA (20 μM) and incubated in the dark for 30 min. The fluorescence intensity was evaluated by a flow cytometer based on a comparison with fluorescence excitation (485–495 nm) and emission (525–530 nm).

Evaluation of mitochondrial membrane potential (ΔΨm)

MMPs (ΔΨm) levels were determined by Rhodamine 123 staining. MDA-MB-231 cells were treated with IC_{50} concentrations of ethyl acetate and methanol extracts for 24 h and incubated with 400 μL of Rhodamine 123 (50 μM) at 37 °C for 30 min. In order to removing extracellular Rhodamine 123, the cells were washed twice with PBS. The fluorescent intensity of the Rhodamine 123 was assessed by flow cytometer with excitation 488 nm and emission 525-530 nm. The mean fluorescence intensity represented the cellular levels of intracellular MMP.

Spheroids cell culture and treatment

In attempt to three-dimensional (3D) spheroids production two particular methods (hanging drop and liquid onerlay) were used and developed.

Hanging drop

The hanging drop method was used to form the MDA-MB-231-sphroids. 50 μL of prepared cell suspension (3×10^3 cells/drop) was inserted into the lid of the 25 mm tissue culture dish; then the lid was smoothly inverted onto the bottom of dish which was filled with 3 ml of PBS and incubated for 4 days at 37 °C in a 90% humidified incubator with 5% CO_2. The drops maintain in place due to surface tension.

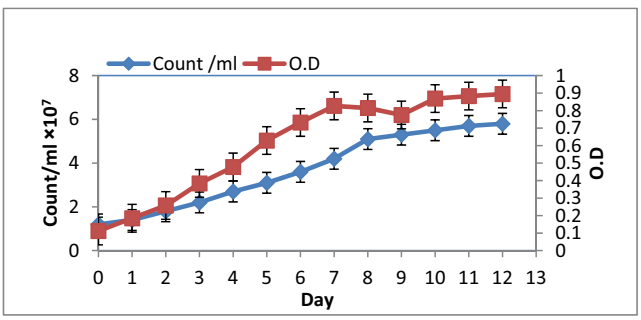

Fig. 1 Growth curve of *Picochlorum* sp. RCC486

Table 1 The growth parameters of *Picochlorum* sp. RCC486

Parameters	At the time of inoculation	Stationary phase
pH	7.78	7.98
Optical density at 620 nm	0.112	1.178
Dry weight (g/L)	0.29	1.279
Cell Density ($\times 10^7$ cells mL^{-1})	0.65	3.32
specific growth rate (μ day^{-1})	–	0.435

Liquid overlay

As increasing spheroids size and for treatment, the liquid overlay method was conducted. After 4 days incubation, spheroids were sorted into a 96 well U bottom plate which was precoated with 0.5% poly-HEMA (sigma) and air dried for at least 3 days prior to use. The plates were incubated at 37 °C and 5% of CO_2 incubator. Cells were then exposed to different concentrations ranged between 100–500 μg/mL of methanol extract for 48 h. After treatment spheroids of each well were transferred to a flat bottom 96-well plate, supernatant was removed and 200 μL/well of MTT solution (1 mg/mL) in phenol red-free medium was added and the plate was incubated at 37 °C for an additional 4 h. After removing the supernatants DMSO was added and spheroids incubated on a shaker at 37 °C until crystals were completely dissolved. Finally, absorbance was recorded at using ELISA multi-well plate reader (Gen5, Epoch, BioTek, America) at 492 nm.

Statistical evaluation

Values were presented as mean values plus or minus the standard deviation (SD) of three independent experiments. Statistical differences between the means of the treated and untreated cells were determined by Student's test, and $p < 0.05$ was accepted as being statistically significant.

Results

Isolation and identification of the new *Picochlorum* strain

The new marine microalgae, isolated from Persian Gulf, is a small, circular, and non-flagellated cell of about 2–3 μm in diameter. The strain was identified based on molecular studies. Amplification of the chromosomal DNA of the new strain with SSU850 (F) and SSU870 (R) universal primers, yields a single band. After DNA sequencing, a fragment with 1310 bp of the 18S rRNA encoding gene was determined. The sequence of this region was published in the Iranian Biological Resource Center (IBRC) database under accession number KT861323 and submitted to Basic Local Alignment Search Tool (BLAST) analysis. The isolated sequence displayed very high percentage of identity with published 18S rDNA sequence of the *Picochlorum* sp. RCC486 from chlorellaceae family.

Growth analysis

The specific growth rate of the strain (μ) *Picochlorum* sp. RCC486 was studied based on the slope of logarithmic plots which was obtained from growth curves. The optical density was read at 620 nm. The results were shown in Fig. 1 and revealed that *Picochlorum* reached the log phase by 12 days. Different parameters such as pH, DW, and OD, were recorded and analyzed at the incubation time and at the stationary phase. The data were illustrated in Table 1.

Total phenol content

The total phenolic content of different solvent extracts (hexane, chloroform, ethyl acetate and methanol) of microalgae *Picochlorum* sp. RCC486 were evaluated and reported in Fig. 2. The methanol extracts had the most phenolic content which was 3.39 ± 0.05 mg/g DW gallic acid equivalent and the lowest phenolic content was related to hexane extract with 1.52 ± 0.09 mg/g DW gallic acid equivalent.

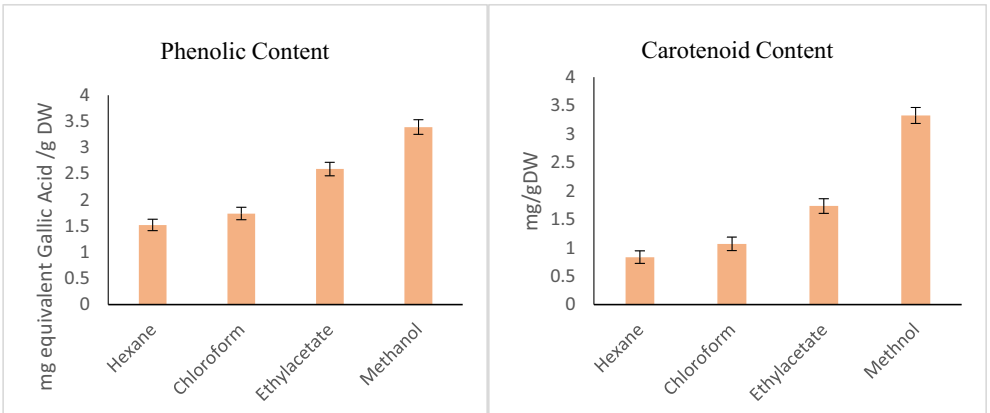

Fig. 2 Total phenolic and carotenoid content of hexane, chloroform, ethyl acetate and methanol of *Picochlorum* sp. RCC486

Table 2 In vitro cytotoxic activities (IC_{50} μg/mL) of four different extracts against four cancer cell

Extracts	MDA-MB-231	MCF-7	Hep-G2	A549	HDF
Methanol	147.65 ± 0. 12	240.61 ± 0.60	283.04 ± 0.50	404.13 ± 0.19	>5000
Ethyl acetate	191.76 ± 0.50	254.29 ± 0.50	340.86 ± 0.70	514.39 ± 0.18	>5000
Chloroform	>500	>500	>500	>500	>5000
Hexane	>500	>500	>500	>500	>5000
Etoposide	20.3 ± 0.21	22.08 ± 0.39	17.4 ± 0.49	16.58 ± 0.78	92.7 ± 1.2

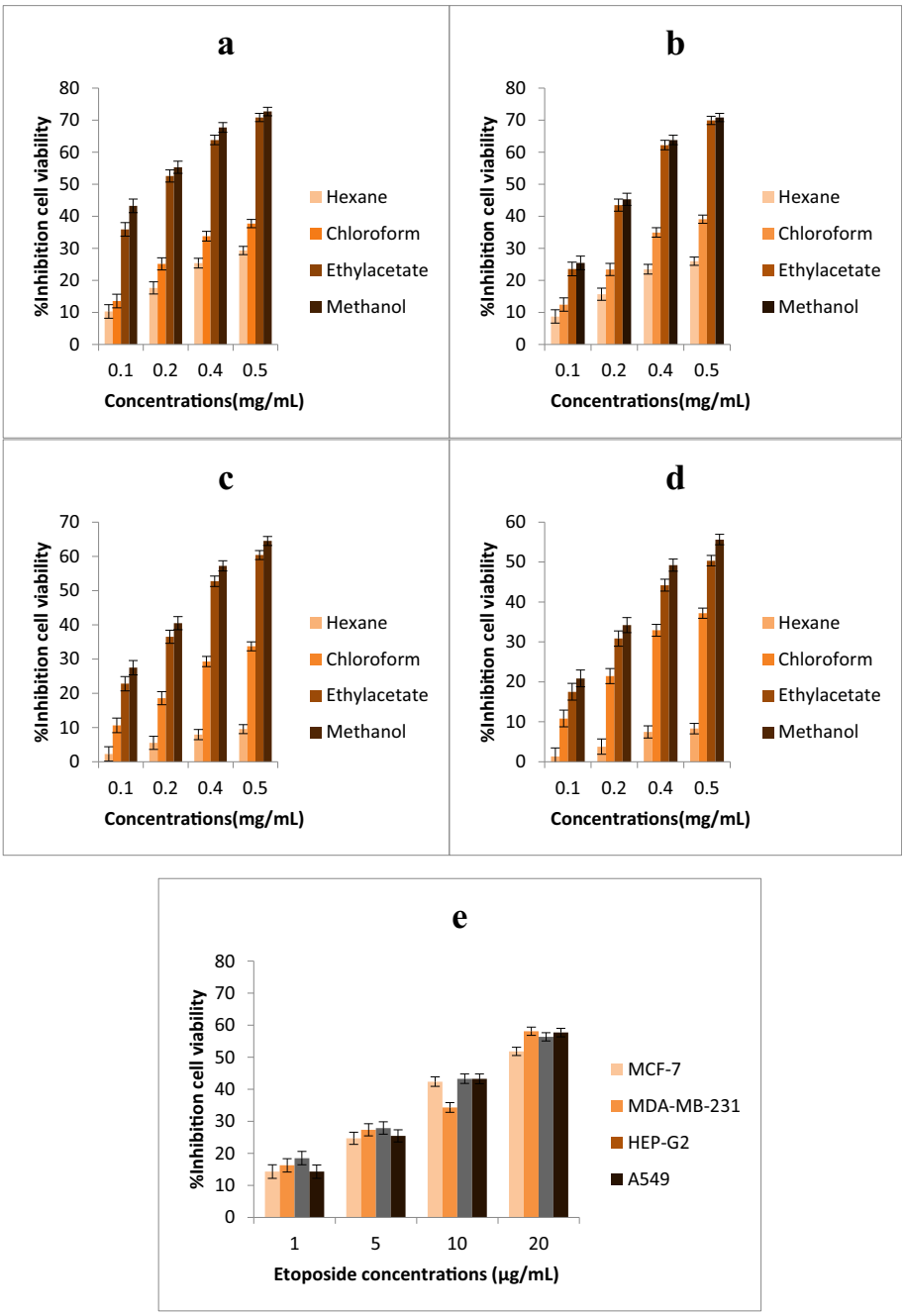

Fig. 3 The Inhibition effects of the hexane, chloroform, ethyl acetate and methanol extracts on (**a**) MDA-MB-231, (**b**) MCF-7, (**c**) Hep-G2, and (**d**) A-549. The etoposide as positive control was evaluated on cancer cell lines (**e**). The cancer cells were treated with four different extracts at 0.1, 0.2, 0.4, and 0.5 mg/mL concentrations of the extracts and 1, 5, 10, and 20 μg/mL. The data shown are the Mean ± SD from three independent experiments, each with triplicate wells

Carotenoid content

Carotenoid content of hexane, chloroform, ethyl acetate and methanol extracts of microalgae *Picochlorum* sp. RCC486 were shown in Fig. 2. Methanol extracts showed the highest carotenoids content (3.33 ± 0.06 mg/g) while hexane extracts had lowest of this content (0.83 ± 0.04 mg/g).

Growth inhibition and cell viability

The in vitro cytotoxic activity of hexane, chloroform, ethyl acetate, methanol extracts and etoposide as positive control drug was determined by MTT colorimetric assay. The experiment was conducted by four different concentrations of the extracts including 0.1, 0.2, 0.4, and 0.5 mg/mL against particular human cancer cell lines, including MDA-MB-231, MCF-7, Hep-G2, and A549. The results of the MTT assay showed that the methanol extract exhibited most cytotoxic activity against all cancer cell lines. In this case, the IC_{50} values against MDA-MB-231, MCF-7, Hep-G2, and A549 were $IC_{50} < 0.5$ mg/mL. Among the tested extracts methanol extract showed the highest cytotoxic activity against MDA-MB-231 with IC_{50} value of 0.14 mg/mL. Moreover the ethyl acetate extract showed a significant growth inhibitory activity against all four cancer cell lines especially MDA-MB-231 with IC_{50} value of 0.19 mg/mL. While the chloroform and hexane extract had no significant cytotoxic activity against all cancer cell lines ($IC_{50} > 0.5$ mg/mL). The concentrations of extracts were raised up to 5 mg/mL against HDF. The IC_{50} of the extracts against human normal cell line was higher than 5 mg/mL. The IC_{50} values were shown in Table 2 and the concentration-dependent inhibition of extracts presented in Fig. 3.

The cytotoxicity of methanol extract was assessed by treating MDA-MB-231 3D spheroids with various concentrations of the extracts for 48 h .The IC_{50} value of the methanol extract on MDA-MB-231 spheroids was 482.09 µg/mL. Because of less sensitivity of solid tumors models to chemotherapeutics, the IC_{50} value of methanol extract on spheroids have become approximately 3 time more than that of monolayer cell culture (Fig. 4).

Acridine orange (AO)/ethidium bromide (EB) double staining analysis

The viability and morphological changes in treated cells was assessed by AO/EB double staining method. AO penetrates both viable and dead cells and emits green fluorescence while EB is taken up only by dead cells and emits red fluorescence after DNA intercalating. MDA-MB-231, MCF-7, and Hep-G2 cell lines were treated with IC_{50} dose of ethyl acetate and methanol extracts, and DMSO1% as negative control. The

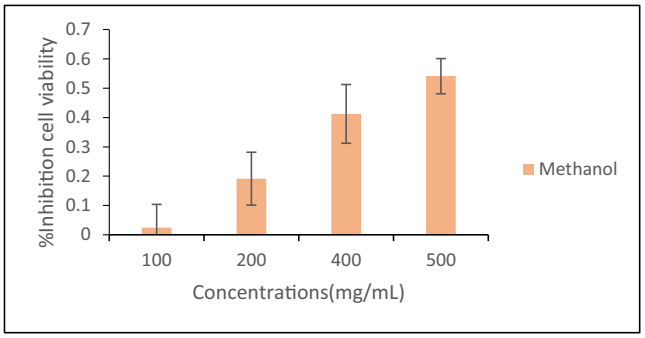

Fig. 4 The Inhibition effects of methanol extract on 3D spheroids of MDA-MB-231. Spheroids were treated with different concentrations of methanol extract for 48 h. The data shown are the Mean ± SD from three independent experiments, each with triplicate wells

morphological changes of cells were evaluated by fluorescence microscopy. Analysis of the fluorescence microscope images (Fig. 5) revealed that the cells which were treated with the ethyl acetate and methanol extracts of *Picochlorum* sp. RCC486 showed morphological changes with respect to the control cells.

Annexin V-FITC/PI flow cytometry assay

Annexin V-FITC/PI staining assay was conducted to determine early and late phase of apoptosis and necrosis in treated cancer cell lines. Flow cytometry analysis was performed on MDA-MB-231 and Hep-G2 cell lines using the Annexin V-FITC/PI double staining after being incubated with IC_{50} concentrations of ethyl acetate and methanol extracts for 24 h. The results confirmed apoptosis induction in a significant proportion of treated cells. As illustrated in Supplementary Fig. 6, in MDA-MB-231 cells ethyl acetate and methanol extracts caused apoptosis in 33.2 and 33%, respectively. The percentage of apoptosis induction by ethyl acetate (23.4%) and methanol (28.9%) extracts in Hep-G2 cells were quite lower than that of MDA-MB-231 cells. Also, there wasn't any significant percentage of necrosis in all treated and control cells.

Caspase-3 activation assay

Caspase-3, a cysteine aspartate-specific protease, is a key effector in the process of apoptotic cell death. Caspase-3 colorimetric assay was conducted in MDA-MB-231 cells to finding out whether the induction of apoptosis by ethyl acetate and methanol extracts is dependent on caspase-3 activity or not. The caspese3-specific substrate (acetyl-Asp-Glu-Val-Asp) is labeled with the chromophore p-nitroaniline (pNA). Activation of caspase-3 could lead to cleavage of the caspase-3 specific substrate and subsequently, the chromopher pNA releases. MDA-MB-231 cells were treated with IC_{50} concentration of ethyl acetate and methanol extracts and

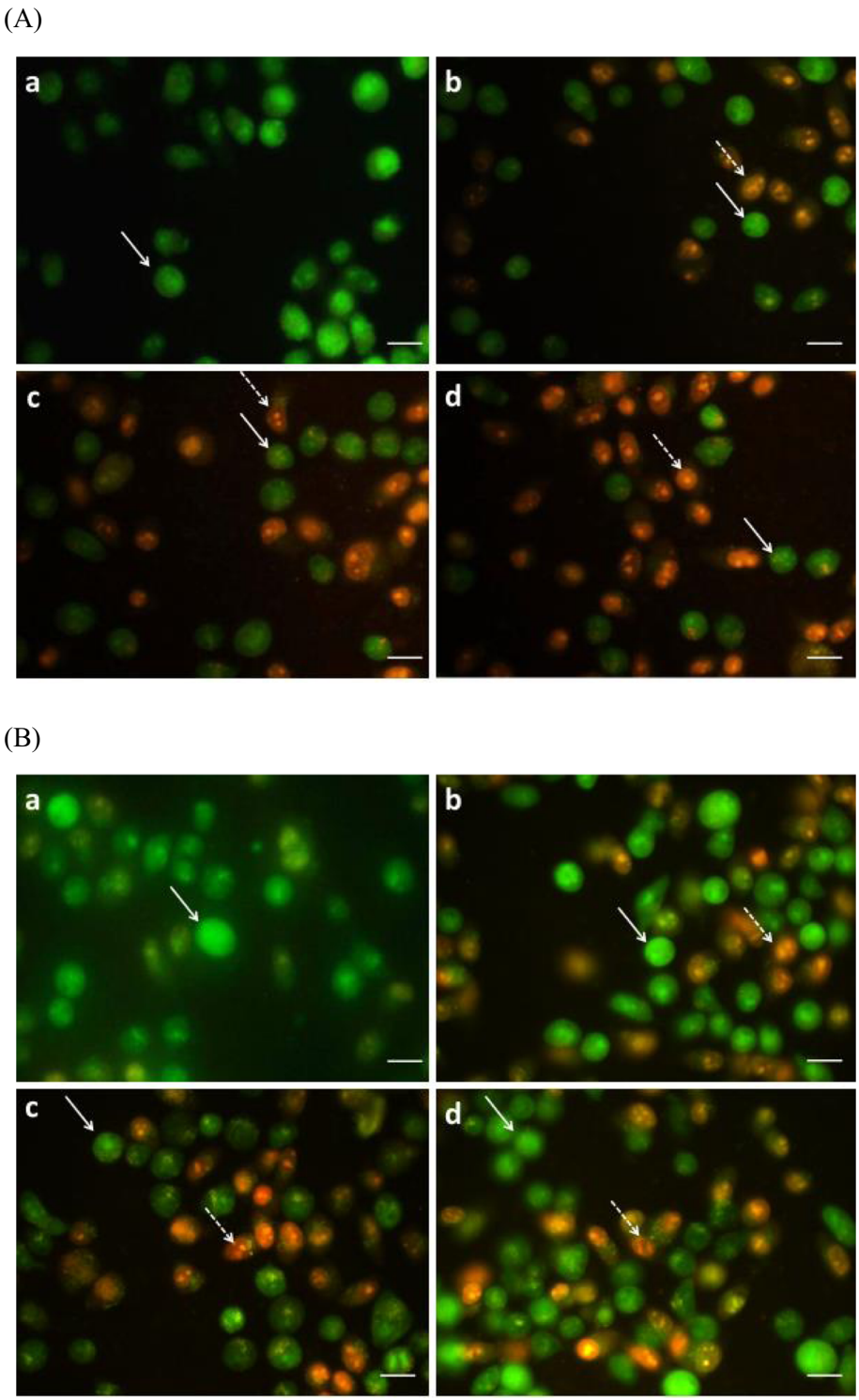

Fig. 5 Fluorescent microscopy analysis of the (**A**) MDA-MB231, (**B**) Hep-G2 and (**C**) MCF-7 cells stained with acridine orange/ethidium bromide. a: DMSO 1% as negative control, b: cells treated with IC_{50} of etoposide c and d: cells were treated with IC_{50} of the ethyl acetate and methanol extracts. White arrow indicates live cells and dashed arrow shows apoptotic cells. The images were taken with a fluorescence microscope at 40 ×

analyzed by a spectrophotometer at 405 nm. The results showed a significant 3.5 to 4 fold increase in caspase-3 activity in treated cells relative to the control (Fig. 7). The data suggested that the cytotoxic activity of ethyl acetate and methanol extracts in MDA-MB-231 cells occurs via caspase-3-dependent apoptosis.

(C)

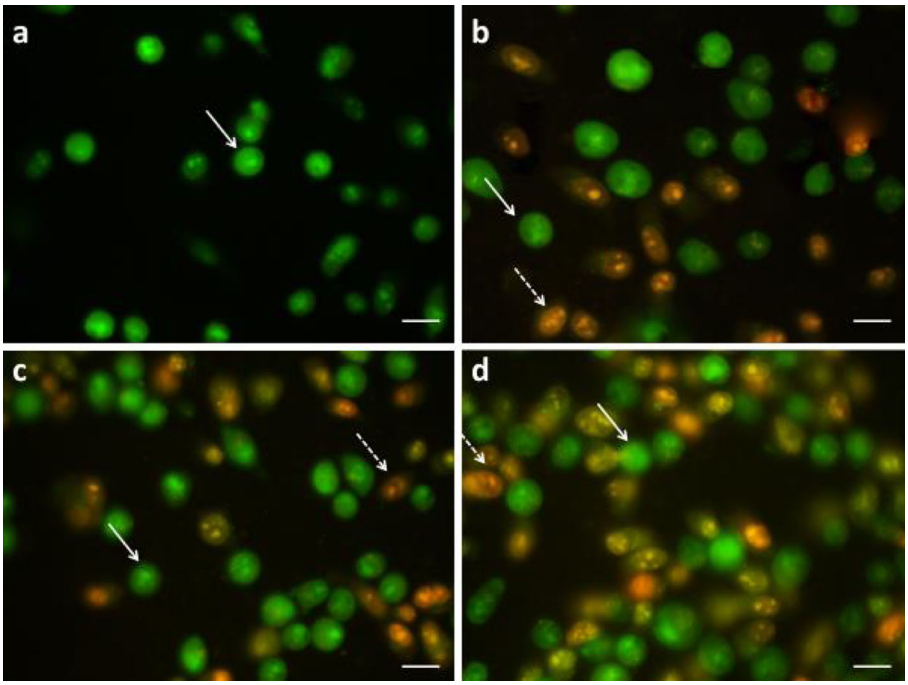

Fig. 5 (continued)

Measurement of intracellular reactive oxygen species (ROS)

According to increasing evident ROS induced by apoptotic stimuli could lead to mitochondrial dysfunction and intrinsic apoptosis pathway activity. So we hypothesized that most potent extracts may have caused apoptosis in MDA-MB-231 cells via increasing ROS production. In an attempt to know the association between ROS production and apoptosis induction, we measured the level of ROS generation after 24 h treatment with the IC_{50} concentration of ethyl acetate and methanol extracts and DMSO1% as negative control. Treated cells were incubated with DCFH-DA (ROS-specific fluorescent dye) and the fluorescence intensity was determined using flow cytometer. The results confirmed that ROS level increased rapidly after treatment with ethyl acetate and methanol extracts in respect to the control. Fluorescence intensity of DCFH-DA shifted right in treated cells and the percentage of DCF-positive cells was about 45 and 51% after exposure to ethyl acetate and methanol extracts, respectively (Supplementary Fig. 8).

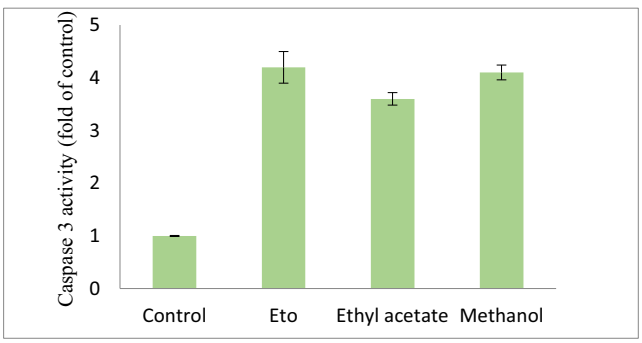

Fig. 7 The effect of ethyl acetate and methanol extracts of *Picochlorum* sp.RCC486 on caspase-3 activation in MDA-MB-231 cell line. Caspase-3 activation were determined by adding DEVD-*p*NA to the cell lysates and monitoring colorimetric substrate hydrolysis at 405 nm using spectrophotometer. The values are shown as a fold-increase compared to the control

Mitochondrial membrane potential (ΔΨm)

ROS augmentation may oxidize mitochondrial pores and therefore mitochondrial function may be impaired. Disruption of the mitochondrial membrane could lead to apoptogenic factors releasing and apoptosis induction. In this experiment, we used Rhodamine 123 staining to evaluate the effect of ethyl acetate and methanol extracts on mitochondrial-dependent apoptosis in MDA-MB-231 cells. Rhodamine123 is a fluorescence dye and can be absorbed by mitochondria. After 24 h treatment with DMSO1% (as negative control) and IC_{50} dose of ethyl acetate and methanol extracts, MDA-MB-231 cells were stained by Rhodamine 123 and their MMP were evaluated by flow cytometer (Supplementary Fig. 9). The results revealed a significant reduction of MMP ($\Delta\Psi m$) after treatment with IC_{50} concentrations of ethyl acetate (9.81%) and methanol (11.5%) extracts of the *Picochlorum* sp. RCC486 in comparison with untreated cancer cells

(37.5%). The results indicate that the mitochondrial apoptosis pathway may have involved in apoptosis-induced by extracts.

Discussion

Cancer disease is the second cause of death and responsible for about 13% of fatalities in the world. The anti-cancer drugs currently used, show undesirable effects and because of that, it seems searching for new effective and safer dugs is quite necessary [15]. One of the approaches for finding and developing new drugs is screening crude extracts. In the present study, the anti-cancer activity of *Picochlorum* sp. RCC486 extract, isolated form Persian Gulf, have been evaluated against MDA-MB-231, MCF-7, Hep-G2 and A549 cancer cell lines.

Results of MTT assay revealed that the methanol and ethyl acetate extracts have a significant anti-proliferative activity with strongest inhibition (lowest IC_{50} values) against all the human cancer cell lines but not against human normal cell line. The results suggest these extracts contains molecules with anti-cancer properties that may act on cancer cells; while the other two extracts (chloroform and hexane) showed negligible cytotoxic activity against all tested cancer cell lines due to lack of anti-cancer properties molecules.

One of the goals of this study was to validate the cytotoxicity activity of most potent extracts on 3D models of MDA-MB-231 cells. The conventional 2D monolayers, are routinely used for evaluating the effectiveness and safety of agents with potential as anti-cancer drugs, as initial model systems [16]. Since we get the best performance of apoptosis induction by methanol extract in MDA-MB-231, we choose them for 3D cell culture and MTT assay. The result of MTT assay on MDA-MB-231 spheroids revealed that the IC_{50} value of methanol extract on spheroids have become approximately 3 time more than that of monolayer cell culture. So the present study revealed that spheroids were more resistant to the toxic effects of the most potent extract compared to 2D cultures. It has been believed spheroids can more realistically mimic tumor behavior more effectively than 2D cell cultures. Treatment responses in 3D cultures may be different from 2D due to several differences: altered gene expression, quiescent cells and hypoxic areas inside of spheroids, insufficient penetration of drugs into the core of spheroid, and other tumor-like features [17].

Analysis of the AO/EB staining revealed that methanol and ethyl acetate induced a significant apoptosis in MDA-MB-231, Hep-G2 and MCF-7 cell lines. On the other hand, AnnexinV-FITC/PI staining showed high early apoptosis induction by methanol and ethyl acetate extracts in tested MDA-MB-231 and Hep-G2 cell lines.

We showed that highly polar methanol and ethyl acetate extracts contain high phenol and carotenoid contents as opposed to chloroform and hexane extracts. Previous study have shown that although microalgae extract containing higher phenolic compounds could induce more growth inhibition and apoptosis in human liver cancer cells [18]. Also, considerable evidences suggest that some carotenoids extracted from microalgae such as *Chlorella ellipsoidea* and *Chlorella vulgaris* are able to interfere with the molecular pathways of cancer and could change the expression of apoptosis proteins and ultimately induce apoptosis in cancer cells [19]. It has been revealed that antioxidant can exhibit prooxidant behavior under certain condition. The prooxidant activity of phenolic compounds is depended on presence of redox-active transition metals, pH, and high phenolic concentration. Reduced transition metal ions are more soluble and stable in acidic pH and could induce prooxidative reaction [20–22]. Microenvironment in tumors is generally more acidic than in normal tissues because of anaerobic condition and consequently the high production of lactic acid. Rising in amount of lactic acid leads to acidic environment which has been found in many tumors [23]. This acidic pH could enhance prooxidant activity of polyphenols and could help prooxidative reactions.

Also, in other study, researchers proved that poly phenol compounds such as curcumin, gallotannins, and resveratrol have cytotoxic activity against cancer cells via mobilization of endogenous cooper and they have prooxidant activity by this mechanism. Apoptosis inducing properties of polyphenolic compounds, such as binding and cleavage of DNA and the generation of ROS in the presence of transition metal ions are similar to those of known anticancer drugs [24]. Phenoxyl radicals which are produced from phenolic compounds have prooxidant behavior. A reaction between phenoxyl radicals and oxygens could lead to generation of H2O2, O2 and a complex combination of quinones and semiquinones [20]. There are evidences which are suggested that some drugs or natural compounds which can enhance the intracellular H2O2, could be useful as anticancer drugs [25].

Carotenoids such as β-carotene could show prooxidant activity in high dose of carotenoids and high pressure of oxygen. The higher pressure of oxygens lead to the higher prooxidant activity of carotenoids because of autooxidative processes [20]. In this process, a reaction between carotenoid radical and oxygen will be happened and ultimately carotenoid peroxyl will be produced which has prooxidant activity. There are evidences which are suggested that prooxidative process is increased in the presence of unsaturated lipids [26]. It should be mentioned that a majority of researches about prooxidant activity of natural antioxidants have been done on in vitro condition and prooxidant activity of this compounds in in vivo condition should be evaluate in future studies [20].

Considering the result of previous experiments, the effect of methanol and ethyl acetate on caspase-3 activity, ROS production and MMP were measured in MDA-MB-231 cells.

There was a significant increase in activation of caspase-3 after exposure to the extracts which was fully consistent with

the results of previous studies. As it mentioned, the methanol and ethyl acetate extracts have a high content of phenolic and carotenoid compounds. The data achieved form previous studies indicate that phenolic compounds like phenolic acid and carotenoids compounds like β-carotene could induce apoptosis thorough caspas-3 activation. It has been demonstrated that the activation of caspase3 followed by activation of caspase-8 and caspase-9, after cell exposure to β-carotene occur [27, 28].

Our data confirmed that ROS level increased significantly in treated MDA-MB-231 cells and subsequently, depolarized MMP increased too. It has been proved that phenolic and carotenoids compounds could enhance ROS production and free radicals like ROS could change the inner mitochondrial membrane and also could open mitochondrial permeability transition pore. Opening the pore may lead to loss of MMP and releasing some of pro-apoptotic molecules into cytoplasm. Binding the pro-apoptotic molecules like Bax and Bid to the outer membrane of mitochondria is a signal for releasing the internal content of mitochondria and ultimately, thorough signaling pathways the apoptosome complex formation will be happen [29].

Previous studies showed that prooxidant compounds could enhance the production of ROS level which could have cytotoxic effect in cancer cells but not in normal cells due to higher concentration of cooper ions and high metabolic activity in cancer cells as opposed to normal cells [20]. Our results confirmed that methanol and ethyl acetate extracts enhanced the production of ROS in cancer cell line but not in human normal cell line.

Other compounds with anticancer activity have been recognized in microalgae. May be the anticancer activity of our microalgae is related to those compounds. For example natural pigments (NPs) like chlorophyll showed a great anticancer activity in some studies [30]. It has been reported that natural stroles like stigmasterol have antiproliferative effect on cancer cells and could induce apoptosis. The evaluation of cytotoxic activity of stigmasterol which was isolated form a kind of microalgae upregulated pro-apoptotic gene expression such as Bax and P53 and downregulated antiapoptotic gene such as Bcl-2 [31]. The effect of polysaccharides on growth inhibition of cancer cells has been evaluated in some studies. These polysaccharides were dived from a type of marine microalgae and the results confirmed their anticancer activity via inhibition of topoisomerase I and II, and consequently induction growth inhibition in cancer cells [32]. In addition to all of these mechanisms some studies revealed that there are a number of antioxidant with anti-cancer activity which acting through epigenetic mechanism like DNA demethylation or histone modification. Overall, our study supports a pharmacological role for *Picochlorum* sp. RCC486 methanol and ethyl acetate extract as a candidate for cancer therapy.

Conclusion

In particular, this study showed that *Picochlorum* sp. RCC486 as a marine microalgae species could induce apoptosis in some cancer cell lines via caspase-3 activation, high level ROS production and MMP reduction. It seems that further studies should be focused on secondary metabolites of the methanol and ethyl acetate extracts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

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