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Research Article

Evaluation of anticancer effect of *Cladophora glomerata* algae extract

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Abstract
Cladophora glomerata has numerous biologically active properties and is considered one of the most essential medicinal algae. The purpose of this research was to investigate the anticancer efficacy of *Cladophora glomerata* algae extract against human hepatocellular carcinoma (HepG2), human cervical carcinoma (HeLa), and normal mouse embryonic fibroblast (MEF) cell lines. The needed algae was found in the Hassan Al-Hamoud River , Baquba, Iraq. The chemical detection of some chemical components of the ethanolic extract of *C. glomerata* revealed that the extract contains a group of active compounds. The study showed significant variation ($p<0.05$) among inhibition percentages of cancer cell line HepG2, Hella, and MEF cell lines that were treated with different concentrations (15.1, 31.2, 62.5, 125, 250, 500, and 1000) µg/ml of *C. glomerata* extract. The outcomes showed that increased inhibition percentages of the above cell line were associated with increased concentrations. The inhibition percentage of HepG2, Hella, and MEF cell lines were 1.6 ± 30.88, 1.7 ± 14.10, and 1.2 ± 2.31 at concentration 15.1 (mg/ml), and was 3.8 ± 84.90, 3.6 ± 88.29, and 3.9 ± 23.2, respectively at concentration 1000 (mg/ml). The study concluded that the *C. glomerata* extract with different concentrations significantly inhibited cancer cell lines (HepG2 and Hela) and ME because they have antiproliferative and antioxidant activity against cancers. The extract's inhibitory impact begins at low doses and increases with increasing concentration. The study would be beneficial to use macroalgae as new and sustainable sources of bioactive compounds against cancer.

Keywords: *Cladophora glomerata*, Hepatocellular carcinoma (HepG2), Human cervical cancer (Hela), Mice embryo fibroblast (MEF)

INTRODUCTION

Algae are a diverse group of photosynthetic organisms found in both fresh and saline water.Algae produce a wide variety of metabolites, which assist algae in adapting to the harsh conditions of the marine environment. Researchers hunting for medicinally essential medications, particularly those with anticancer effects, have recently shown a heightened interest in these molecules due to the diversity and singularity of their structures (Hannan *et al*., 2020).

Cancer has long been one of humanity's most severe physical, economic, and psychological threats. The detection and treatment of cancer has been a major concern for medicine practitioners for a very long time. Consequently, new pharmaceuticals are consistently required to support medical professionals in oncology treatments (Hanahan, 2022).

Cladophora is a group of macroscopic green algae that includes more than 183 species and lives either in freshwater or salt water (Michalak and Messyasz, 2021). *Cladophora glomerata* is a pervasive filamentous alga in freshwater environments and lakes. Also in deep water, nutrient-rich, shallow water. *C. glomerata* possesses numerous biologically active properties and is regarded as one of the most essential medicinal algae, among other applications .Numerous studies have demonstrated its efficacy in cosmetics and nutrition as an antibacterial, antifungal, and anti-inflammatory agent. It also has anti-diabetic, anti-oxidation, and anti-cancer properties.

This moss contains abundant flavonoids, alkaloids, phenols, tannins, fatty acids, sterols, and terpenoids. (Al-Jaber *et al*., 2021). An increase in the amount of

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nutrients (nitrogen and phosphorus) present in the water is primarily responsible for the appearance of this annual filamentous macroalgae. This nutrient enrichment results from intensive farming practices such as using fertilizers containing minerals and detergents including phosphorus. Additionally, this nutrient enrichment results from expanding human populations, wastewater treatment plants, and other factors (Zhou *et al*., 2019). Polysaccharides, polyphenols, terpenes, carotenoids, and phycobiliproteins are some of the anticancer bioconstituents present in algae (Munir *et al*., 2019).

In human oral cancer cell lines derived from the KB strain of the disease, it is shown that the ethanolic extracts of *C. glomerata* exhibit powerful anti-tumour activities. (Laungsuwon and Chulalaksananukul, 2013). Previous studies have demonstrated that the green algae *C. glomerata* can be utilized as a safe factory for creating AgNP due to the presence of a number of compounds that contribute to the reduction and stability of the AgNP. This potential was discovered as a result of the existence of the aforementioned chemicals. The tumor-fighting agents or anti-cytotoxic impact suggests that this method may be useful for treating tumors by preventing the growth of cancer and tumour cells and resolving the issue of medicine resistance. This effect suggests that this technique may be effective for treating cancers. (Fayyad *et al*., 2022). The biogenic silver nanoparticles generated from the algae *C. glomerata* were evaluated on the HCT-116 cell lines (Acharya *et al*., 2022).

A methanolic extract of *C. glomerata* containing alkaloids has been demonstrated to effectively prevent cancer cell proliferation MCF7 (Al□Jaber *et al*., 2021). A methanolic extract of *C. glomerata* contains high levels of antioxidants, including flavonoids and phenols, and has been shown to reduce stress caused by oxidation and enhance the viability and mitochondrial effectiveness of equine adipose-derived stem cells from mesenchymal tissues (Sundaramoorthy *et al*., 2022). The purpose of the present study was to examine the anticancer effect of *C. glomerata* against cancer cell lines, including hepatocellular carcinoma (HepG2) and human cervical carcinoma (Hela), as well as the control Mice embryo fibroblast (MEF) cell line.

MATERIALS AND METHODS

Algae samples collection

Algae samples were collected from 12 stations including water ponds, rivers and streams from different areas of the city of Baquba in Diyala Governorate in the fall of 2022, using a planktonic collection net with holes of 20 microns in diameter. The samples were kept in tightly closed and sterilized plastic bottles, with each sample's date and collection area confirmed.

Methods

Samples examination

The samples were examined using a light microscope mounted at 40x and 100x magnifications to identify the algae collected during the modeling process. The approved classification source was used to classify algae (Prescott, 1973) and identified the location of the moss required in the study and it was in the Hassan Al-Hamoud River near the entrance of the College of Engineering/University of Diyala. The algae identified was *Cadophora glomerata,* belonging to family Cladoporaceae

Preparation of extract from *Cladophora glomerata*

The dried algae is ground in an oven at 50°C for an hour with a blender and turned into a powder. The powder (10 g) was filled into a thimble and extracted with (150 mL) 70% ethanol, using a Soxhlet apparatus at 60°C for 9 hours. (John Wiley & Sons *et al*., 2011). The extract was oven dried at 38–40 °C. Then, the dry extract was collected and kept at 4ºC in the refrigerator until the standard solution was prepared and the concentrations used for this study were 15.1, 31.2, 62.5, 125, 250, 500, and 1000 µg/ml. To determine the primary chemical constituents of the methanolic extract of *C. glomerata*. These analyses included tests for alkaloids, phenols, triterpenes, and sterols according to Harborne (984), tannins according to Jawad (1997), saponins according to Haddad (1965), and glycosides according to Cannell (1998) and flavonoids according to Jaffer *et al*. (1983).

Cancer cell lines used

Maintenance and cultivation of cell lines

The cancer cell lines used in the study were prepared by the Iraqi Center for Cancer Research and Medical Genetics in Baghdad, Iraq .The were produced using the technique described by Fresheny (2015). Each line's cells were grown in a 25 cm2 culture dish containing RPMI-1640 medium and 10% Fetal Calf Serum (FCS). Plates containing the cell suspension and culture medium were incubated in an incubator for 24 hours at 37°C and 5% CO2. After one day of incubation, the cells were examined under an inverted microscope to ensure their growth, viability, and absence of contaminants. The procedure of washing the cells with PBS solution and discarding them was repeated twice. Then, a trypsin-versene solution was added to the cells and incubated for 30 to 60 seconds at 37°C, followed by a serum-containing culture medium to inhibit the enzyme. They were collected in centrifugal containers to precipitate the cells and centrifuged at room temperature at 2000 rpm/min for 10 minutes. The filtrate was discarded, and the cells were suspended in a new culture medium with 10% serum. The number of cells was determined by adding a specific volume of Trypan Blue

stain to the same volume of the cell suspension and using a Hemacytometer slide to determine the vitality of the cells.

Cytotoxic assay

The toxic effect of the ethanolic extract of *C. glomerata* was tested in the cancer cell lines (HepG2 , Hela and MEF) cell line by adding the solution of trypsin-versene to the plate of tissue culture (with a size of 25 cm^2). Twenty ml of the RPMI-1640 was added to the Fetal Calf Serum (FCS) and mixed. Then 0.2 ml of the mixture was added to every 96 wells in a plate of tissue culture by using a micro-pipette. The plates of tissue culture were incubated for 1 day at 37°C in an incubator until the cell adhesion to the wells of the plate. Then the medium was removed from the wells and 0.2 ml from each of 15.1, 31.2, 62.5, 125, 250,500,1000 µg/ml concentrations of the extract were added with three replicates for each. The negative control was prepared by using three replicates of dimethyl sulfoxide (DMSO), while the positive control was prepared by adding three replicates of PBS supplemented with 0.2 ml of serum-free media and kept in the incubator for 24 hours at 37°C according to (Fresheny, 2015). Then the plates were taken out of the incubator and
disposed of the dead cells and old culture media from the holes. After that, 100 µl of crystal-violet dye was added to each well, and the plates were re-incubated for 20 minutes. The contents were removed and washed using PBS to remove the remnants of the dye, and then the cells were left to dry. At a wavelength of 492 nm, the data were read off using a spectrophotometer. The following equation was used to determine the rate at which cell growth could be inhibited:

Percentage of inhibition rate (IR) % = (X - Y \ X) x 100 Eq.1

Since:

X = Reading (O.D) of control

Y = Reading (O.D) for each concentration treatment

Statistical analysis

Data of the present study is described as Mean±SD. The differences among means of inhibitions of cell lines by *C. glomerata* extract were measured by Duncan test (ANOVA). SPSS version 25 program was used to programmed these data and at a significant level $P \leq 0.05$.

RESULTS AND DISCUSSION

Chemical detections of some active compounds in an extract *C. glomerata*

In the results of the chemical examination, it was found that there are many biologically active compounds, including alkaloids and flavonoids, Tannins, saponins, glycosides, triterpenoids, and sterols are found in *C. glomerata* and were free of phenols.

Effect of *Cladophora glomerata* extract on HepG2, Hella, and control cell line MEF.

A cytotoxicity test was performed on three cancer lines (Hepg2, Hela, and MEF) to determine the effect of *C. glomerata* extract on the growth of cancerous tumors. At 37°C for 24 h, cancer cell lines were treated with seven concentrations of the extract: 15.1, 31.2, 62.5, 125, 250, 500, and 1000 g/ml, with three replicates for each concentration. A cytotoxicity assay was used to measure the effect of extract concentrations on cell growth as a percentage of growth inhibition.

Table (1) showed that the extract had an inhibitory effect on the growth of cancer cells for the HepG2 cell line, starting with a concentration of 15.1 µg/ml, where the inhibition rate reached 30.88%, and this percentage increased to reach 36.48%, 49.40%, 53.32%, 60.11%, 66.51%, and 84.90 % for concentrations of 31.2, 62.5, 125, 250, 500, and 1000 µg/mL, respectively. No significant difference was observed between concentrations of 15.1 and 31.1 µg/mL and between 250 and 500 µg/mL concentrations. Fig. 1 compares untreated HEPG2 cells and those treated with the crude extract at a 1000 µg/ml concentration.

Table 2 shows that the extract had an inhibitory effect on the HeLa cancer line, which started with a concentration of 15.1 µg/ml, as the inhibition rate reached 14.10% and increased to 28.86%, 41.20%, 56.90%, 75.96%, 78.67%, and 88.29% for concentrations 31.2. , 62.5, 125, 250, 500, and 1000 µg/mL, respectively. No significant difference was observed between 250 and 500 µg/ml concentrations. Fig. 2 compares untreated HELA cells and those treated with the crude extract at a 1000 µg/ml concentration.

Table 3 showed that the extract affected the normal MEF line, but it had a lesser effect compared to the inhibition on the cancerous lines above, as the percentage of inhibition reached 2.31%, 6.54%, 9.64%, 10.2%, 10.97%, 11.46%, and 23.2% for concentrations 15.1. ,

Table 1. Inhibition percentages in the cancer cell line HepG2 by the effect of different concentrations of the extract for 24 hours of exposure at a temperature of 37 °C

Inhibition±SD	Concentration (mg/ml)
1.6 ± 30.88 e	15.1
1.1 ± 36.48 e	31.2
2.9 ± 49.40 d	62.5
2.9 ± 53.32 c	125
3.1 ± 60.11 b	250
3.5 ± 66.51 b	500
3.8 ± 84.90 a	1000

Small different letters refer to significant differences at p<0.05

Table 2. Inhibition percentages in the cancer cell line Hella by the effect of different concentrations of the extract for 24 hours of exposure at a temperature of 37 °C

Inhibition±SD	Concentration (mg/ml)
1.7 ± 14.10 f	15.1
2.2 ± 28.86 e	31.2
2.6 ± 41.20 b	62.5
3.0 ± 56.90 c	125
3.1 ± 75.96 b	250
3.4 ± 78.67 b	500
3.6 ± 88.29 a	1000

Small different letters refer to significant different at p<0.05

Table 3. Inhibition percentages in the control cell line MEF by the effect of different concentrations of the extract for 24 hours of exposure at a temperature of 37 °C

Inhibition±SD	Concentration (mg/ml)
1.2 ± 2.31 d	15.1
1.8 ± 6.54 c	31.2
1.9 ± 9.64 b	62.5
2.5 ± 10.2 b	125
3.1 ± 10.97 b	250
3.3 ± 11.46 b	500
3.9 ± 23.2 a	1000

Small different letters refer to significant different at p<0.05

31.2, 62.5, 125, 250, 500, and 1000 µg/mL, respectively. No significant difference was observed between 62.5, 125, 250, and 500 µg/ml concentrations. Fig. 3 compares untreated H cells and those treated with the crude extract at 1000 µg/ml concentration.

Based on the statistical results, it was found that there was a significant difference when comparing the effect of the extract on cancer cell lines after using the IC50 concentration (300) for the HepG2 line and the HeLa line (100). This is the same for the regular MEF line (1000), as shown in Table (4).

Concentration values of IC_{50} in HepG2, HELLA and MEF cell lines

As shown in Table 4, the present outcomes showed the IC_{50} of *Cladophora* extract scored highest level at MEF cell line (1000 mg/ml) and lowest level at Hella cancer cell line (100 mg/ml).

The utilization of molecules of oxygen as a final oxidant in the process of respiration confers major benefits to aerobic organisms. Even though oxygen is an entirely harmless molecule, it can undergo a partial reduction, which results in the production of dangerous reactive oxygen species(ROS), such as superoxide radicals (O;) radicals of hydroxyl (OH) and peroxide hydrogen (H2O2). Each of these reactive oxygen species has the potential to interact with a variety of biomolecules that are located inside the cell. This presents a persistent oxidative risk to the biological functions inside the cell. The alteration of a biological process within a cell due to reactive oxygen species is called oxidative stress. (Cheung and Vousden, 2022).

The accumulation of mutations in the sequence of nucleic acids is caused by the cellular damage that is produced as a result of oxidative stress (OS) caused by free radicals. Accumulated mutations in the DNA play a role in the beginning and progression of a wide variety of severe diseases, including cancer, rheumatoid arthritis, various heart disorders, Parkinson's illness, Diabetes, Alzheimer's disease, and a host of others. These mutations can also play a role in the ageing process (Dumanović *et al*., 2021).

Additionally, numerous environmental stresses, including, but not limited to, high temperatures, direct sun-

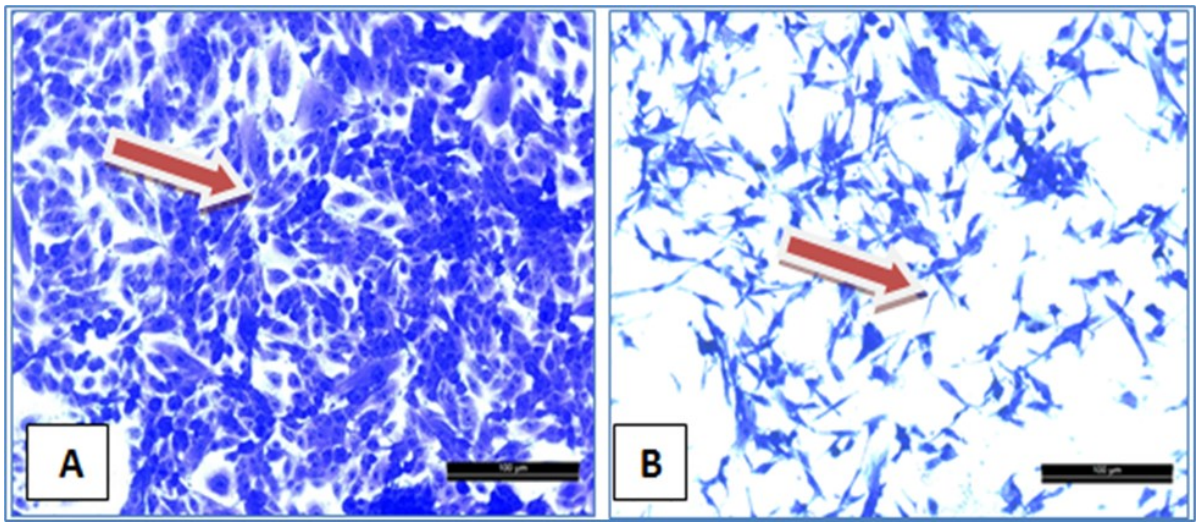

Fig. 1. *Comparison between untreated HEPG2 cells and those treated with crude extract at 1000 µg/ml concentration for 24 hours exposure at 37 °C (100X) using Crystal Violet dye ;*A) HEPG2 cancer cell line representing control, showing dense cells, B) HEPG2 cell line treated with extract at 1000 µg/ml, showing dead cells and intercellular spaces.

Table 4. **C**oncentration values of IC50 in HepG2, HELLA and MEF cell lines

IC_{50} (mg/ml)	Cell lines
300	HEPg2
100	Hella
1000	MEF

light, insecticides and pesticide toxic metals, high salinity, pollutants, ultraviolet (UV) rays, a wide variety of infectious pathogens, and so on, all contribute significantly to free radical production. (Sakuma *et al*., 2018).
To counteract the effects of oxidative stress, living cells have evolved many enzymatic and non-enzymatic antioxidant defense systems. Superoxide dismutase (SOD) is a subset of unique enzymes that reduce highly reactive oxygen molecules.
(Ezejiofor and Orisakwe, 2019). The link between oxidative stress and cancer prevalence is becoming increasingly clear in medical research with each passing year. (Ikwegbue *et al*., 2017). Antioxidant supplementation by physicians has become standard clinical practice. Antioxidant supplements sold over the counter are becoming increasingly popular among people in general (Urso and Clarkson, 2003). Research conducted in vitro and in vivo studies have shown that using naturally occurring antioxidant compounds can significantly reduce the risk of developing malignancy by preventing the formation of free radical compounds, preventing the progression of DNA damage and the development of mutations in genes and proteins (Sundaramoorthy *et al*., 2022).
It has been demonstrated that a number of the compounds that are derived from marine algae have substantial anticancer action against a variety of cancer cell lines. Through a mechanism involving PI3K and Akt, apoptosis was induced in gastrocancer cells by polysaccharides that were extracted from *Capsosiphon fulvescens* (Li *et al*., 2009). *In vivo* research conducted by Han et al. (2022) demonstrated that certain extracts of the marine algae *Thalassia testudinum* could suppress the proliferation and progression of colon cancer. It has been found that the mechanisms behind this effect are the inhibition of angiogenic activity, the activation of autophagy, and the development of immune system responses against tumors. The anticancer and antimetastatic effectiveness of the marine chemical penisuloxazin A has been shown by Zhang et al. (2021) in an *in vitro* breast cancer model. The turnover of epithelial-mesenchymal transition (EMT) and C-terminal suppression of heat shock protein Hsp90 have been proposed as mechanisms for this impact.
Researchers found that *C.glomerata* preparations have potent antioxidant properties and superoxide scavenging properties in marine environments. The SOD dismutase activity was highest (98.250.93 U/mg protein) in the marine algae Chloroform extract. Scientists argue that marine algae have developed formidable antioxidant defenses to cope with increasing pollution and marine contamination (Sundaramoorthy *et al.,* 2022).
The algae *Cladophora glomerata* extract exhibits anticancer action against HT29 colon cancer cell lines because it has an antiproliferative impact on this cell line (Sundaramoorthy *et al*., 2022). *Cladophora glomerata* has compounds that enhance nanoparticles' stability and reduction characteristics. It may be employed as a safe, local, and natural source for AgNP production and a safe factory for many other metal nanoparticles. The phycosynthesized AgNPs have anticancer and antitumor effects in MCF-7 and L20B cell lines, indicating their potential for use as a trend in cancer nanotherapy. Furthermore, *C. glomerata* plays a significant function in treating giardiasis patients. (Fayyad *et al*., 2022).

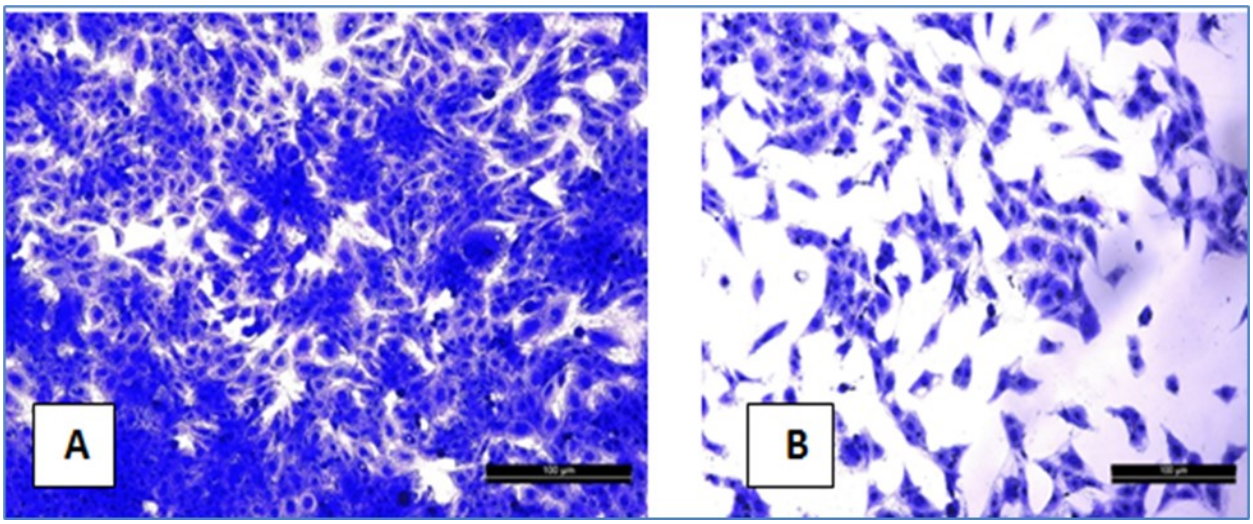

Fig. 2. *Comparison between untreated and treated Hella line cells with crude extract at 1000 µg/ml concentration for 24 hours exposure at 37 ºC (100X) using Crystal Violet dye; (A) Hella cancer cell line is the control, showing dense cells, B) HELLA cell line treated with extract at a concentration of 1000 µg/ml, showing dead cells and intercellular spaces*

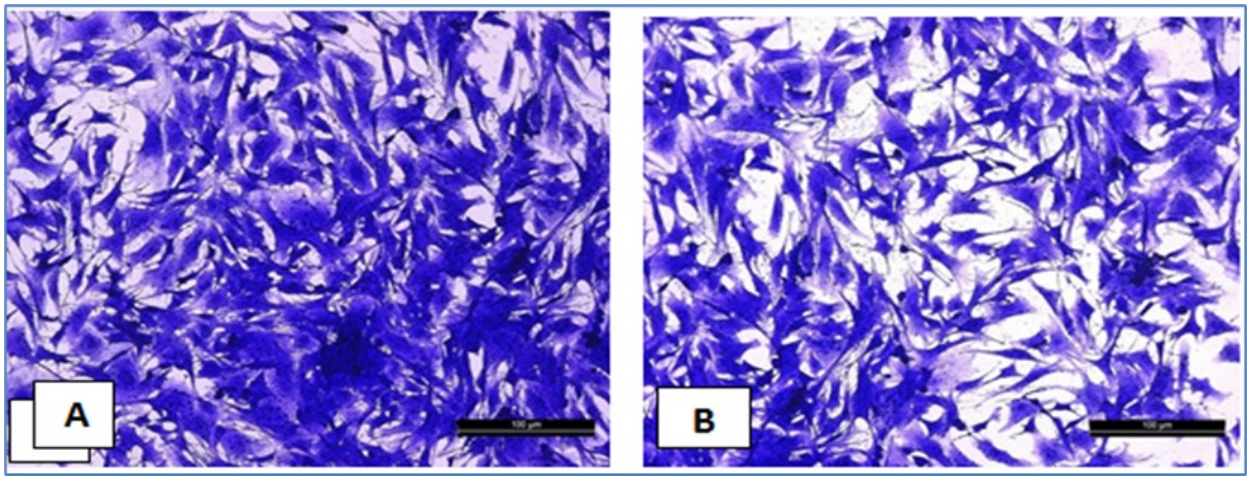

Fig. 3. *Comparison between untreated MEF line cells and those treated with crude extract at 1000 µg/ml concentration for 24 hours exposure at 37 °C (100X) using Crystal Violate dye*; *A) MEF cancer cell line, which is the control, showing dense cells, B) MEF cell line treated with extract at a1000 µg/mL concentration, showing dead cells and intercellular spaces.*

Because two cytotoxic and selective compounds, loliolide and epi-loliolide, were isolated, the researchers say that crude extracts and/or biomass of the marine algae *Tisochrysis lutea* can be a source of compounds for the prevention and treatment of human hepatocarcinoma (HEPG2). These compounds are probably degradation products of fucoxanthin and/or zeaxanthin (Gangadhar *et al*., 2020). Carotene isolated from the marine red alga *Gracillaria sp.* inhibited the growth of human hepatocellular carcinoma (HepG2) cells. This was accomplished by modulating multiple molecular pathways, which included blocking the activation of intracellular growth signaling proteins Akt and ERK1/2 and down-regulating the expression of endogenous antioxidant enzymes SOD-2 and HO-1, as well as its transactivation factor, Nrf-2. (Kavalappa *et al.,* 2019). A recent study showed the *P. pavonica* extract exhibited the highest inhibition against HepG2 cell growth due to its highest antioxidant property and highest total polyphenol contents (TPCs) (positive correlation between antioxidant and total polyphenol contents) (Goutzourelas *et al*., 2023).

Majumder *et al.* (2015) showed the antiproliferative activities of *C. glomerata* extracts against the human cervical cancer cell line, SiHa, and these results nearly to present results that this marine algae plays an important role in the inhibition activity of the human cervical cancer cell line, Hela. The present results showed the activity of *C. glomerata* extract with different concentrations in the inhibition of cell lines of hepatocellular carcinoma (HepG2), human cervical cancer (Hela), and Mice embryo fibroblast (MEF). In addition, the present study found that cell line inhibition rates are increased with progressed concentrations of extracts. Based on our acknowledgement, the present results consider the first study that showed the activity of *C. glomerata* against cell lines of hepatocellular carcinoma (HepG2), human cervical cancer (Hela), and mice embryo fibroblast (MEF) .

Conclusion

The present study concluded that the *C. glomerata* extract with different concentrations significantly inhibited cancer cell lines (HepG2 and Hela) and normal cell lines (MEF) due to its antiproliferative and antioxidant activity against cancers. The higher concentrations of the extract inhibited more than the low concentrations. Thus, *C. glomerata* algae, possessing active substances, can be used as a safe, local and natural source, particularly against HepG2 HELL cancer cell lines.

Conflict of interest

The authors declare that they have no conflict of interest.

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