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Original Article

Evaluation of anti-oxidant and anticancer effect of marine algae *Cladophora glomerata* in HT29 colon cancer cell lines- an *in-vitro* study

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Abstract: Background: Marine algae are a huge Pandora's box of rich nutrients and huge medicinal compounds. These therapeutic compounds are investigated widely for their anticancer, antioxidant, and anti-diabetic properties. Objectives: This study aims to evaluate the antioxidant and anticancer effect of the marine algae *Cladophora glomerata* (collected from Hare Island-Tuticorin region) on HT-29 colon cancer cell lines. Methodology: The marine algae, *Cladophora glomerata*, was collected, processed, and authenticated. Methanol, Ethyl acetate, Chloroform, and Hexane extracts were prepared using a hot solvent extraction process. These extracts were subjected to SOD assay and MTT assay. 5 Fluorouracil was used as the positive control. Results: The antioxidant activity of the SOD assay was found to be 85.66±0.81, 80.10±1.25, and 98±0.93 U/mg protein for methanol, ethyl acetate, chloroform, and hexane extracts, respectively. L-Ascorbic acid was used as the positive control whose SOD antioxidant value was found to be 139±1.24 U/mg protein. The IC50 value of methanol, ethyl acetate, chloroform, hexane algae extracts, and 5 Fluorouracil against HT29 cell lines was calculated to be 28.46±0.65, 48.56±1.19, 93.7±0.91, 88.53±0.83, and 8.2±1.3 µg/ml, respectively. Conclusion: From the above study, we can infer that the methanol extracts of the algae *Cladophora glomerata* have excellent anticancer activity. Therefore, these compounds can be purified and analyzed further for a potential lead as an anticancer molecule.

Keywords: Anti-cancer, marine algae, pharmacology, in-vitro, MTT assay, superoxide dismutase

Introduction

Algae constitute a group of eukaryotic heterogeneous photosynthetic organisms, mainly found in the marine environment. Algae produce numerous wide ranges of metabolites that help them cope with the harsh conditions of the oceanic milieu. Due to their structural diversity and uniqueness, these molecules have recently gained a lot of interest in identifying medicinally valuable agents, including those with potential anticancer activities [1, 2].

Cancer has always been a substantial health threat to humankind physically, economically, and psychologically. It has always been a massive challenge for medical professionals to diagnose and treat cancer. Therefore, there is always a need for new drugs to help medical professionals in oncology therapeutics [3].

Among the numerous bio compounds present in marine algae, polysaccharides, polyphenols, terpenes, carotenoids, and phycobiliproteins have significant anticancer activity.

Cladophora is a group of ubiquitous, reticulated, filamentous green algae distributed equally in freshwater and marine sources [4, 5].

It has been proved that the ethanolic extracts of Cladophora glomerata have shown significant anti-tumor activities in KB human oral cancer cell lines [6]. Biogenic silver nanoparticles synthesized from the algae Cladophora glomerata has been studied against HCT-116 cell lines [7]. Methanolic extracts from Cladophora glomerata have been shown to inhibit the growth of MCF7 cancer cells [8, 9]. Also due to the rich presence of flavonoids and phenols, the methanolic extract of Cladophora glomera-

ta has shown to possess high antioxidant properties and has shown to decrease oxidative stress and also improve the viability and mitochondrial potential in equine adipose-derived mesenchymal stem cells [10].

In this study, we will examine the antioxidant and anticancer activity of the marine algae *Cladophora glomerata* collected around the shores of Tuticorin, TamilNadu, in HT29 colon cancer cell lines.

Materials and methods

Plant material

Cladophora glomerata was collected from Hare Island, Tuticorin, Tamil Nadu (southeast coast of India). The algae were identified and authenticated in the Department of Algology, Tamil Nadu Veterinary and Animal Sciences University, Fisheries College and Research Institute, Thoothukudi-628008, Tamil Nadu.

Preparation of extract

The seaweed *Cladophora glomerata* collected were washed three times with seawater, then successively with tap water and distilled water to remove the epiphytes, sand, and other wastes attached along with the algae. After thorough washing, they were air-dried under the shade for two weeks and finely powdered using a blender.

The hot solvent extraction process using four different solvents was used to extract the algae material. The solvents were chosen according to different degrees of polarity. They are as follows Methanol, Ethyl acetate, Chloroform, and Hexane.

25 g of the sample was weighed and extracted with 300 ml of methanol, ethyl acetate, chloroform and hexane (with solvents of increasing polarity) by continuous 64 Hot percolations with the help of Soxhlet apparatus for 10 hrs of time. On completion, the extract was filtered and concentrated using a rotary evaporator under reduced pressure and a controlled temperature of 50°C-60°C. The residues were made up to 100 mg/ml of concentration and stored at room temperature [11].

Antioxidant assay

Assay of superoxide dismutase (SOD)

Superoxide dismutase was assayed by standard protocol as described in previous studies [12].

Procedure: 0.5 ml of HT29 colon cancer cell line tissue homogenate treated with the four different algae extracts (i.e. methanol, ethyl acetate, chloroform, and hexane extract) was diluted to 1.0 ml with water, then 2.5 ml of ethanol and 1.5 ml of chloroform were added (chilled reagents).

This mixture was shaken for 90 Sec at 4°C and then centrifuged.

The SOD enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, and 0.3 ml of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 ml.

The addition of 0.2 ml NADH started the reaction. After incubation at 30°C for 90 Sec, the reaction was stopped by adding 1 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with the addition of 4 ml n-butanol. This assay is based on inhibiting the formation of NADH phenazinemethosulphate-nitroblue tetrazolium formazan.

The mixture was allowed to stand for 10 min, centrifuged after that, and then the n-butanol layer was separated.

The color density of the chromogen in n-butanol was measured at 520 nm. The color developed at the end of the reaction was extracted into the N-butanol layer and measured in a spectrophotometer at 520 nm.

L-Ascorbic acid was used as the positive control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit.

In vitro assay for cytotoxicity activity- MTT assay

HT29 colon cancer cell lines were procured from the National Cell Centre, Pune. All culture

Table 1. SOD enzyme activity values of different extracts of the marine algae spp. *Cladophora glomerata*

S.NO	NAME OF THE SAMPLE	SOD (U/mg protein)
1.	Methanol extract of *C.glomerata*	85.66±0.81
2.	Ethyl acetate extract of *C.glomerata*	80.10±1.25
3.	Chloroform extract of *C.glomerata*	98.25±0.93
4.	Hexane extract of *C.glomerata*	76.33±1.05

media and chemicals were purchased from Sigma Aldrich Pvt.Ltd.

This assay was performed according to previous studies [13, 14].

The HT29 colon cancer cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37°C. Cells were fed with fresh cultured medium every 2-3 times per week and subcultured when 80% confluent. All cell cultures were screened for mycoplasma infection.

HT 29 colon cancer cells (1×10^5/well) were plated in 24-well plates and incubated at 37°C with 5% CO_2 condition. After the cell reached the confluence, the various concentrations of the different algae extracts (the test samples) were added and incubated for 24 hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100 µl/well (5 mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals.

After incubation, 1 ml of DMSO was added to all the wells, solubilizing the crystals. The absorbance at 570 nm was measured with a UV-Spectrophotometer using DMSO as the blank. Measurements were performed, and the concentration required for a 50% inhibition (IC50) was determined graphically. The assay was performed in triplicates and with 5 Fluorouracil used as the positive control. The rate of tetrazolium reduction is proportional to the speed of cell proliferation.

The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells/A570 of control cells × 100%

% cell death was calculated using the formula 100% cell viability.

Graphs were plotted using the % of Cell death at the Y-axis and the concentration of the sample on the X-axis. Cell control and sample control are included in each assay to compare the full cell viability assessment.

Results

Assay of superoxide dismutase (SOD)

Among the algae extracts screened for antioxidant activities, the chloroform extract of the algae *Cladophora glomerata* was found to have the highest antioxidant activity level of 98.25± 0.93 U/mg protein. This was followed by methanol extracts of the algae, which had a SOD of 85.66±0.81 U/mg protein. Ethyl acetate extract of the algae exhibited 80.10±1.25 U/mg protein, and Hexane extract of the algae showed 76.33±1.05 U/mg protein (**Table 1**).

Positive control L-Ascorbic acid was found to have 139±1.24 U/mg protein (**Figure 1**).

MTT assay

The IC50 value of methanol, ethyl acetate, chloroform, hexane algae extracts, and 5-fluorouracil against HT29 cell lines was calculated to be 28.46±0.65, 48.56±1.19, 93.7±0.91, 88.53± 0.83, and 8.2±1.3 µg/ml, respectively (**Table 2**). Among the extracts screened for cytotoxic activity against HT-29 colon cancer cell lines, it was found that methanol extracts of the algae *C.glomerata* have the lowest IC50 value of 28.46±0.65 µg/ml. This indicates that at the IC50 dose of 28.46±0.65 µG/ml, the methanol extracts of the algae *C.glomerata* kill 50% of the HT-29 colon cancer cells, which is the lowest among the above-screened extracts.

This is followed by ethyl acetate extract, which exhibited an IC50 value of 48.56 µG/ml, followed by hexane extract 88.53 µG/ml, and the highest IC50 value 93.7 µG/ml was shown by hexane extract of the algae.

5-Fluorouracil was used as the positive control here, whose IC50 value was found to be 8.2±1.3 µg/ml (**Table 2**).

Comparing the IC50 results of the four different extracts of *C.glomerata,* it was found that

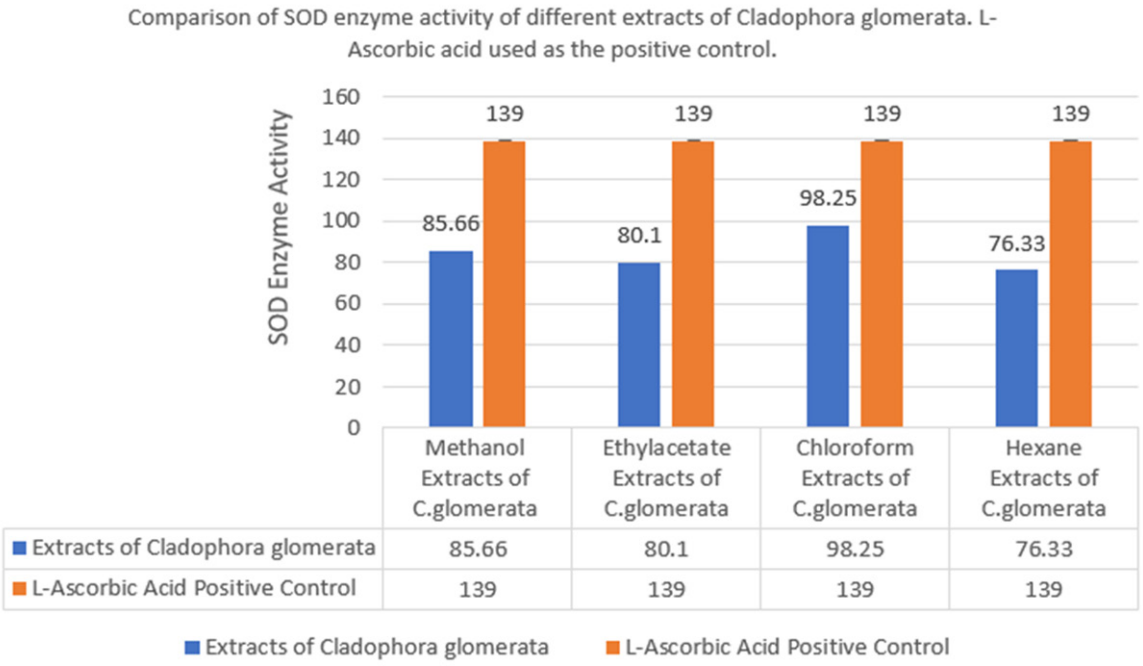

Figure 1. Comparison of SOD enzyme activity of marine algae C.glomerata and L.Ascorbic acid.

Table 2. IC50 values of different extracts of the marine algae *Cladophora glomerata against* HT29 colon cancer cell lines

S.NO	NAME OF THE SAMPLE	IC50 Values (µg/ml)
1.	Methanol extract of *C.glomerata*	28.46±0.65
2.	Ethyl acetate extract of *C.glomerata*	48.56±1.19
3.	Chloroform extract of *C.glomerata*	93.7±0.91
4.	Hexane extract of *C.glomerata*	88.53±0.83

methanol extracts of *C.glomerata* have the lowest IC50 value of 28.46±0.65 µg/ml against HT 29 cell lines (**Figure 2**).

Discussion

Aerobic organisms gain significant energetic advantages by using molecular oxygen as a terminal oxidant in respiration. Although O_2 is itself a totally harmless molecule, it has the potential to be partially reduced and form toxic reactive oxygen species, like the superoxide radicals (0;), the hydroxyl radical (OH), and hydrogen peroxide (H_2O_2). All these reactive oxygen species can combine with the various biomolecules present inside the cell. This possesses a constant oxidative threat to the cellular processes that happen in the intracellular milieu. This alteration of the intracellular biological process by the reactive oxygen species is called oxidative stress [15, 16].

The cellular damage caused by the oxidative stress of free radicals initiates the accumulation of mutations in nucleic acids sequences, Cumulations of mutations in the DNA over time play a role in the initiation and acceleration of many burdensome diseases like cancer, rheumatoid arthritis, various heart disease, Parkinson's disease, Diabetes, Alzheimer's disease etc [17-20].

In addition to this, various stressful environmental conditions like increased heat, exposure to harmful pesticides and insecticides, harsh sunlight light, heavy metals, high salinity, pollutants, UV rays, various infectious pathogens, etc. (to name a few) contribute tremendously to the production of free radicals [21-23].

The living cells have evolved several enzymatic and non-enzymatic antioxidant defense mechanisms to combat this oxidative stress. One such enzyme groups are Superoxide dismutase (SOD) which neutralizes even very highly reactive oxygen molecules [24-26].

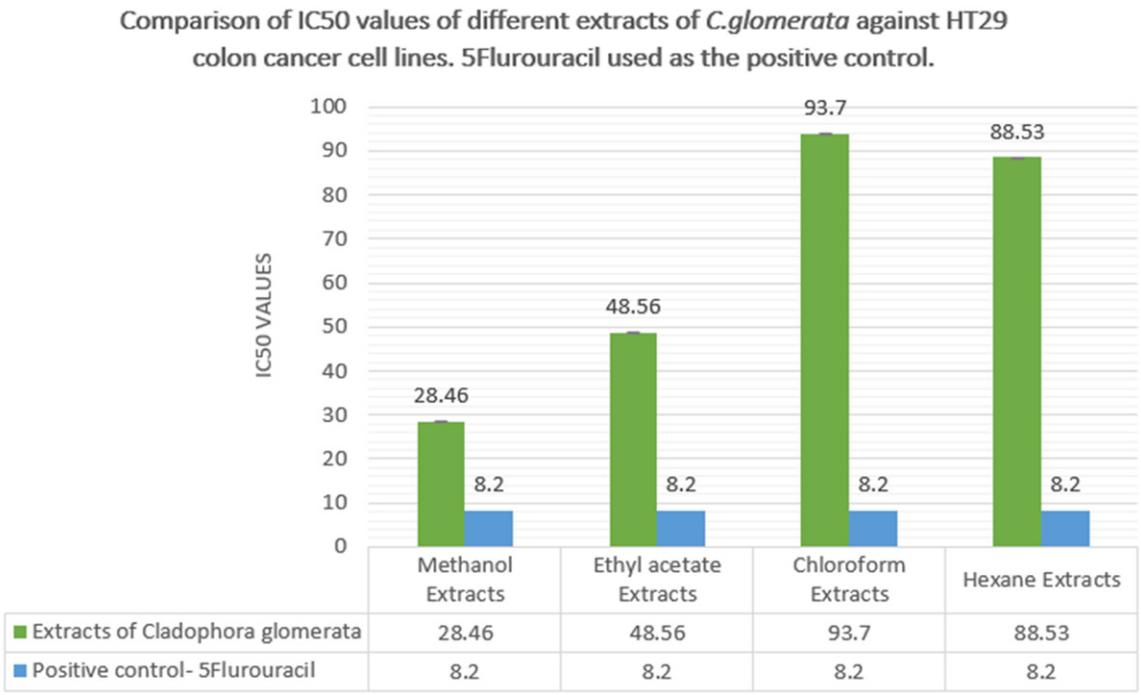

Figure 2. Comparison of IC50 values of various extracts of C.glomerata against HT29 colon cancer cell lines with 5Flurouracil used as a positive control.

SODs are ubiquitous metalloenzymes. The increased levels of superoxide dismutase activity in various aquatic organisms inhabiting harsh marine environments have been proved in many studies [27, 28]. Exposure of the marine algae to various metals resulted in a prominent rise in SOD activity eg.Tetraselmis gracilis with Cd and Gonyaulax polyedra with metal-mixtures of Hg, Cd, Pb, and Cu). Changes in SOD activity were found to be dependent on the metals, their concentration, and the time of exposure [29, 30]. Herbicides also have been proved to have increased SOD activity [31].

From the above study (**Table 1** and **Figure 1**), it can be observed that the different extracts of the marine algae *C.glomerata* have a commendable antioxidant superoxide activity. Among the extracts, the Chloroform extract of the marine algae has the maximum SOD dismutase activity of 98.25±0.93 U/mg protein. We can strongly speculate that to combat the increased pollution and marine contamination, marine algae have evolved strong antioxidant defense mechanisms.

As we all know that chronic inflammatory stress is one of the strong predisposers, that contribute to the pathogenesis of cancer [32]. Thus, the SOD, antioxidant activity of marine algae has a strong contributing role to aiding in the anti-cancer property of the algae as well.

MTT assay was carried out to investigate the inhibitory effects of extracts of green marine alga *Cladophora glomerata* on the growth of human HT29 colon cancer cell lines (**Tables 1**, **2** and **Figures 1**, **2**).

HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology. These cells grow as nonpolarized, undifferentiated multilayers, sensitive to 5-fluorouracil and cisplatin. The MTT is a sensitive in-vitro assay for the measurement of cell proliferation and cell viability. When metabolic events lead to apoptosis or necrosis, there is a reduction in cell viability.

Numerous compounds from marine algae have been proven to show a prominent anti-cancer activity against various cancer cell lines. The polysaccharides extracted from *Capsosiphon fulvescens* via the PI3K/Akt pathway induced the apoptosis of gastric cancer cells [33]. Antiproliferative effect due to cellular apoptosis in oral cancer cell lines was brought about by both

ethanol and methanol extract of the marine red algae *Gracilaria tenuistipitata* [34, 35]. Dimethylsulfoniopropionate, a compound extracted from marine algae exhibited anti-cancer effect in mice with Ehrlich ascites carcinoma [36]. Inhibition of proliferation of human leukemic cell line was observed with the aqueous extracts of Gracilaria corticate and Sargassum oligocystum [37]. Methanol extract of Plocamium telfairiae brought about caspase-dependent apoptosis in HT29 colon cancer cells [38].

From the above study, we can conclude that *Cladophora glomerata* has good antioxidant and anticancer activities. Among the extracts screened, methanol extract of the algae shows promising results against the HT29 colon cancer cell lines and exhibits commendable antioxidant activities to be investigated further for lead molecules. The correlation between oxidative stress and the prevalence of cancer is only gaining stronger evidence with each passing year in the medical literature [32]. The administration of antioxidant supplements by medical professionals has become a routine clinical practice. Over-the-counter Antioxidant supplements are gaining wide popularity among the general public [39]. There is wide invitro and in vivo research emphasizing the prevention of the formation of free radical compounds by naturally occurring antioxidant compounds and thus preventing the occurrence of DNA damage and prevention of formation of mutant gene and protein, thereby considerably lowering the risk of developing cancer [40, 41].

Though these in-Virto studies provide promising results, it's very difficult to narrow down and extract the active individual compound responsible for the anti-oxidant or anti-cancer effect. Khalalide F, a marine-derived cyclic desipeptide was the first marine compound to be isolated from marine algae *Bryopsis Pennata* and to be approved for clinical trials. However, Khalalide F could not proceed beyond Phase 1 clinical studies due to safety concerns. Many synthetic analogs to Khalalide F are being actively developed by researchers and are showing promising antitumor results.

Novel numerous strategies to control tumor progression in symbiosis with the current existing chemotherapy regimen are being actively pursued with great enthusiasm by researchers and Pharmaceutical Industries. Invariably naturally occurring antioxidants with good anticancer activities are attractive targets for lead molecules. Both fresh water and marine algae, with abundant trapped nutrients from the aquatic environment, have always been attractive hot spot for researchers to study the anti-cancer effect.

Limitations

This study has been conducted in HT29 cells only which may provide anticancer effects of extract in only a particular type of colon cancer. Various colon cancer cells have distinctive traits and differ in their metabolism. So extensive research may be recommended for further evaluation in other colon cancer cells like HCT116, SW480, LoVo, etc.

Conclusion

The study results suggest that *C.glomerata* contains beneficial biological compounds that have anticancer activity against HT29 cancer cell lines. However, further studies are needed to confirm and elaborate on the individual compounds that aid in the algae exhibiting anticancer activity.

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Disclosure of conflict of interest

None.

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