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Original Article

ISOLATION AND SCREENING OF MARINE MICROALGAE *CHLORELLA* SP. _PR1 FOR ANTICANCER ACTIVITY

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ABSTRACT

Objective: The objective of the present study includes isolation, characterization and screening of anticancerous activity against B16F10 cell line using isolated marine microalgae *Chlorella* sp._PR1.

Methods: In this study, marine microalgae *Chlorella* sp._PR1 isolated and cultured using f/2 medium and anticancerous activity was assayed using MTT assay.

Results: The DMSO extract of *Chlorella* sp._PR1 was exhibit anticancerous activity against murine melanoma B16F10 cell line. The extract exhibit reduction of cell viability up to 56% with 2µg/ml concentration. IC_{50} were calculated and was found that *Chlorella* sp._PR1 need 5.5 µg/ml of the compounds to reduce the murine melanoma B16F10 cell viability by 50%. Fluorescence activated cell sorting (FACS) analysis revealed that *Chlorella* sp._PR1 extract (8 µg/ml) brought significant inhibition ($p<0.01$) of the G_0-G_1 and the S phase. The extract did not seem to affect the G_2-M phase.

Conclusion: DMSO extract of *Chlorella* sp._PR1 (5.5 µg/ml) was found to be potent against murine melanoma B16F10 cell line.

Keywords: *Chlorella* sp. _PR1, Anticancer, FACS, IC_{50} value.

INTRODUCTION

According to American Cancer Society (Cancer facts and figure 2014) it is estimated that the incidence of cancer in children and adolescent is increased. In 2014, it was estimated that there are 15,780 new cases of cancer and among them 1960 deaths of children and adolescent occur due to cancer. A number of drugs were derived from the natural source such as plant and microorganisms against various different types of cancer (prostate cancer, breast cancer, lung cancer, colon cancer). The marine system consists of indefinite uncharacterized organisms that are producing secondary metabolites for their communication as well as defense purpose [1]. These secondary metabolites also have biological potentials such as anticancerous [2], antimicrobial [3,4], antiviral [5], antidiabetic [6], anti-inflammatory [7] etc. According to literature several anticancerous drugs were isolated from marine sources, among them some are in the clinical trial phase whereas some crossed the clinical phase and now are available in markets [8].

Microalgae are microscopic, and diverse group of eukaryotic organisms that can convert solar energy into chemical energy through photosynthesis. They evolve their own defense mechanisms by secreting toxic secondary metabolite, which is explored in anticancer studies [9,10]. These metabolites have played an important role in pharmaceutical industries. In the present study, marine microalgae were isolated from the Southern Ocean water sample. The marine Microalgae were identified through amplification of ITS region of ribosome. The microalgal extract with different concentration were screened for their anticancerous property conducting of MTT assay.

MATERIALS AND METHODS

Algae isolation and culturing

Microalgae were isolated from the Southern Ocean (Indian sector) water sample. 250 ml of water sample was filtered through the membrane with pore size of 0.22 µm. After filtration, membrane was washed in 5 ml of autoclaved sea water. 30 µl of the above 5 ml were inoculated on 100 µl of f/2 medium [11] and incubated at 25 ^{0}C ± 2 ^{0}C under light (56 µmol m^{-2} s^{-1}). After 15 days, cultures were serially diluted and plated for axenic culture.

Morphological characterization of Microalgae

Morphology of micro algal cells was done through light microscopy by simple wet mount method. The aliquot of each sample was placed in the centre of the clean glass slide and covered with a thin cover slips. The microalgae was examined under light microscope (Olympus, CH20) by 100 × objective lens and photographs were taken. Surface morphology of isolated Microalgae *Chlorella* sp. _PR1 was analyzed through SEM study. The sample was initially fixed with glutraldehyde and post fixed with Osmium tetraoxide. It was then dehydrated with ethanol and allowed to dry completely and was examined under the scanning electron microscope (JSM-6390LV, Jeol, Japan).

PCR Amplification, sequencing and phylogenetic analysis

DNA was isolated by CTAB method and DNA amplification of ITS region of ribosome was carried out using forward (5'-GAAGTCGTAACAAGGTTTCC-3') and reverse (5'-TCCTGGTTA GTTTCTTTTCC-3') primer. Amplification condition was: initial denaturation of 95^{0}C for 4 min., followed by 35 cycle of denaturation at 94^{0}C for 45 sec, 1 min at 60°C as annealing temperature, and extension at 72°C for 1 min and then 10 min final extension at 72ºC. PCR amplification was carried out in GeneAmp PCR 9700 (Applied Biosystem). The PCR product was then purified, sequenced (3130×Genetic analyzer, Applied Biosystem) and analyzed.

Screening for anticancer activity

Culturing of tumor cell line

Highly metastatic murine melanoma cell line B16F10 was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). For subculture, the medium was removed and attached cells were harvested with pre-warmed (37°C) trypsin solution (0.125%). Subsequently, harvested cells were seeded in tissue culture plates with fresh media at 37°C in a humidified 5% CO_2 incubator.

Algal extract preparation

Algal cells were harvested in early log phase and centrifuged at 5000 rpm for 10 min. The pellet was dried and stored at 4C for further

use. 1 mg of dried algal powder of *Chlorella* sp._PR1 was dissolved in dimethyl sulphoxide (DMSO). The selected doses were 2, 4 and 8 µg/ml and the corresponding doses of vehicle control were also tested to observe the effect of DMSO on the cells.

Anticancer assay

Incubation of treated B16F10 cells was carried out in 96-well plates at 37°C for 24 hrs. With various concentrations of microalgal extracts. MTT solution was added to each well (1.2 mg/ml) and incubated for 4hr. Plates were then centrifuged for 10 minutes at 1000 rpm in the cold centrifuge. A volume of 175 µl from the supernatant was pipetted out and subsequently added with 175 µl of DMSO. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 540 nm in an ELISA plate reader.

Statistical Analysis

Statistical analysis was performed with SPSS 10.0 (one way ANOVA followed by Dunett t-test, where the significance level was set at 0.001). Dunett t-test treats one group as a control and treats all other groups against it.

Fluorescent activated cell sorter (FACS) study

2×10^4 B16/F10 melanoma cells, both for treated and control were harvested, washed with phosphate buffer saline (PBS), re suspended in sterile PBS. Cells were fixed in ice cold 100% methanol at l - 20°C for 1 hr. The cells were centrifuged at 5000 rpm for 10 min and the cell pellets were suspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 mg/ml RNase), and incubated at 37°C for 30 min. Subsequently Propidium Iodide (PI) solution (50 mg/ml) was added, and the mixture was allowed to stand for 1 hr in darkness. Fluorescence that emitted from the PI–DNA complex was quantitated after excitation of the fluorescent dye by (FACS Calibur with sorter, BD, San Jose, CA, USA, Fig. A) using Cell Quest software (BD, San Jose, CA, USA) equipped with a 488 nm argon laser and a 525 ± 10 nm band pass emission filter. Fluorescence was captured on a FL2H channel with linear amplification.

RESULTS AND DISCUSSION

Microscopic study of Microalgae via light and scanning electron microscopy

Isolated Microalgae were examined with light microscope 100 × magnification and it was found that they are unicellular, size varied from 3-5 micron and the chloroplast was cup shaped (figure 1a). Surface morphology of marine isolate *Chlorella* sp._PR1 is single cell size of (6.23 µm × 4.51 µm) (figure 1b).

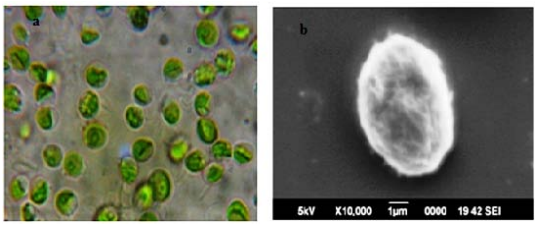

Fig. 1: Morphological study of marine isolate *Chlorella* sp._PR1 a) Light microscopy at 100 × magnification; b) Scanning Electron Microscopy at 10,000 × magnification

Phylogenetic analysis

Multiple sequence alignment (MSA) of the ITS sequences was aligned by CLUSTALW embedded in MEGA 5.0. The phylogram (Figure 2) provided the confirmation that the isolated Microalgae belonged to *Chlorella* sp. Based on sequence homology and phylogenetic analysis *Chlorella* sp._PR1 belongs to *Chlorella vulgaris* (Accession no. 51095187).

GC content of *Chlorella* sp._PR1 is 56.7%. Genes located in low GC region (55-65% GC) shows evidence of shorter intron and less baised codon usage relative to the higher GC region (Hershkovitz & Zimmer, 1996).

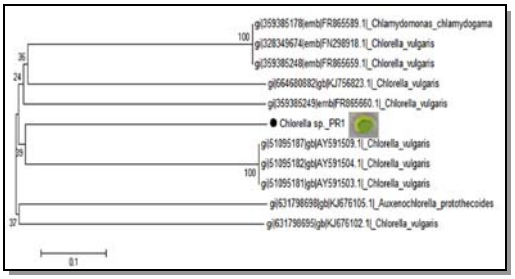

Fig. 2: Phylogenetic analysis based on ITS region of isolated algal strain (*Chlorella* sp. _PR1) and other sequences already deposited and available at NCBI.

Anticancerous assay

The cytotoxicity assay revealed that the microalgal extracts reduced the viability of B16F10 cells in a dose and time dependent manner. The doses 2, 4 and 8 µg/ml and the corresponding doses of vehicle control were also tested to observe the effect of DMSO on the cell viability. A representative figure of cell morphology of B16F10 cell line with and without treatment has been shown in Figure 3.

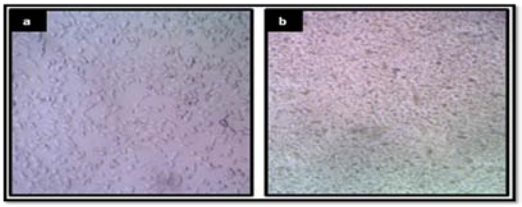

Fig. 3: Morphology of murine melanoma cell line B16F10 (a) without any treatment; (b) after treatment of *Chlorella* sp._PR1.

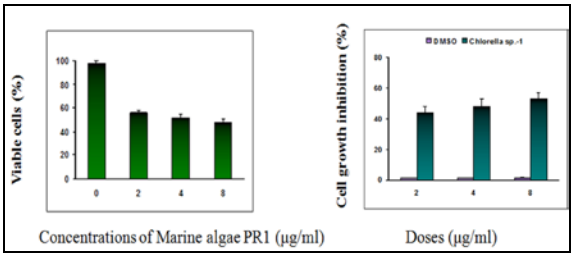

Fig. 4: Inhibition of cell growth in B16F10 cells with *Chlorella sp._PR1.* Dose dependent reduction of cell viability with *Chlorella* sp._PR1 [*p<0.001]; Significant increase in inhibition of cell growth with *Chlorella* sp._PR1 [*p<0.001] in comparison to the corresponding dose of vehicle control.

The extract of *Chlorella* sp._PR1 exhibited a sharp reduction [*p<0.001] of cell viability to 56% with 2 µg/ml after which the increase in the dose of the microalgal extract was associated with a gradual decrease of cell viability (Figure 4). The inhibition of cell growth was significantly more with the *Chlorella* sp._PR1 extract [*p<0.001] in comparison to the vehicle control (Figure 4).

A similar study was done with hexane extract, ethyl acetate extract of *Chlorella ovalis* against HL-60 and murine melanoma B16F10 cell line and was found that, hexane extract effectively suppress the growth of HL-60 and B16F10 compared to control [12]. Two monogalactosyl diacylglycerols (MGDG) induces apoptosis in two genetically-matched immortal mouse epithelial cell lines [13]. The inhibition of cell growth was significantly more with the *Chlorella* sp._PR1 extract [*p<0.001] in comparison to the vehicle control.

IC_{50} of the microalgal extracts

The IC_{50} values of *Chlorella* sp._PR1 extracts have been calculated from the cytotoxicity studies. This was found that *Chlorella* sp._PR1 need 5.5 µg/ml of the compounds to reduce the murine melanoma B16F10 cell viability by 50%.

FACS analysis

In the present study, the murine melanoma B16F10 cells were treated with different doses (2, 4, 8 µg/ml) of *Chlorella* sp._PR1 extract for 24 hrs and were consequently subjected to FACS analysis.

The data obtained from FACS analysis revealed that the *Chlorella* sp._PR1 extract induced an apoptotic effect on the B16F10 cells in a dose dependent manner (Figure 5, 6). The comparative graphical representation depicts the significant rise ($p<0.01$) in the percentage of cells in the sub G_1 phase of the cell cycle with increase in dose of the *Chlorella* sp._PR1 extract compared to the control cells without any treatment (Figure 5). The highest dose (8 µg/ml) brought significant inhibition ($p<0.01$) of the G_0-G_1 and the S phase. The extract did not seem to affect the G_2-M phase.

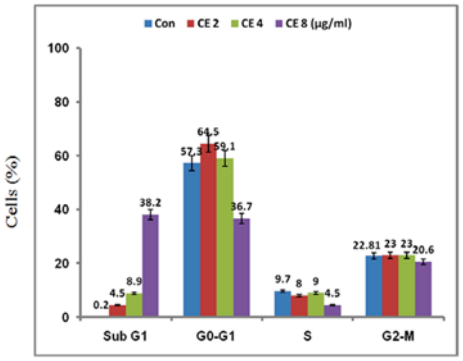

Fig. 5: Comparative representations of the cells (%) at different stages of the cell cycle with various doses of *Chlorella sp._PR1*. Extract (CE). The extract showed a significant dose dependent increase in the percentage of sub G_1 cells (*$p<0.01$) in comparison to the control cells. Highest dose of CE (8 µg/ml) significantly inhibited G_0-G_1 and S phase (*$p<0.01$) of B16F10 cells.

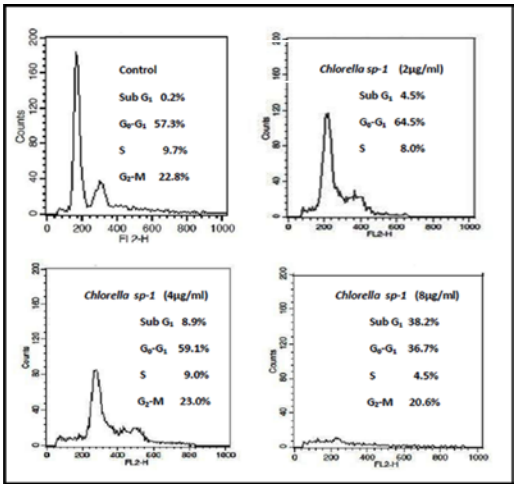

Fig. 6: FACS analysis showing dose dependent apoptotic effect of *Chlorella* sp._PR1 extract on cell cycle of B16F10 cells as evidenced by an increase in sub G_1 peak.

CONCLUSION

In this study *chlorella* sp._PR1 was isolated from the Southern Ocean water sample (Indian sector). *Chlorella* sp._PR1 was found to be potent against murine melanoma B16F10 cell line with 5.5µg/ml

CONFLICT OF INTERESTS

Declared None

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