Cross Current International Journal of Medical and Biosciences
Abbreviated Key Title: Cross Current Int J Med Biosci
ISSN: 2663-2446 (Print) & Open Access

OPEN ACCESS

Volume-2 | Issue-2 | Feb-2020 |

Research Article

Evaluation of Cytotoxic Potentials of Microalgae on Hela, Hepg-2, HT-29 and EACC Cells to Develop Anticancer Agents

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Received: 08.01.2020
Accepted: 26.01.2020
Published: 15.02.2020

Abstract: Finding more effective methods to treat cancer remains a challenge, and development of new therapeutic agents for cancer treatment is essential for continued progress against the disease. This study was conducted to evaluate the in vitro antiproliferative activity of methanolic extracts of *Chlorella vulgaris* and *Nostoc muscorum* against Human cervical Cancer cells (HeLa), liver hepatocellular cells (HepG-2), Human colorectal adenocarcinoma cells (HT-29) and Ehrlich Ascites Carcinoma Cells (EACC) in order to determine the bioacitivities of microalgae. Morphological changes of cancer cells treated with the selected extract were observed by phase contrast microscopy. The cells were incubated with increasing concentrations of microalgal extracts for 24, 48, and 72 h, and cell viability was determined by the Sulforhodamine B (SRB) assay. After treatment of cancerous cells with microalgal extract for 48 h, 55% of the cells were viable, 38% were in early apoptosis, 5% were in late apoptosis, and 1% exhibited necrosis. Further studies are needed to discover the mechanisms underlying the antiproliferative activity of microalgal extracts against cancer cell lines and to identify specific bioactive compounds that are responsible for the observed activities.
Keywords: Microalgae, cytotoxicity, anti cancer, carcinoma.

INTRODUCTION

Cancer includes a large group of pathologies related to the unrestrained proliferation of cells in the body. There are more than 200 different types of cancers, and some cancers may eventually spread into other tissues causing metastases that are often lethal. Cancer is the leading cause of death globally, largely due to aging and growth of the world's population. According to the European Cancer Observatory, estimates for the four most common types of cancer in the European Union in 2012 were as follows: 342,137 cases of colon cancer, 309,589 cases of lung cancer (including trachea and bronchus cancer), 358,967 cases of breast cancer and 82,075 cases of skin melanoma. Finding more effective methods to treat cancer remains a challenge, and development of new therapeutic agents for cancer treatment is essential for continued progress against the disease. According to Dyshlovoy and Honecker (Dyshlovoy and Honecker, 2015) approximately 60% of the drugs used in hematology and oncology have their origin in natural sources, and one third of the most sold are either natural compounds or derivatives thereof.

Today, the pipeline from the initial demonstration that a molecule may have therapeutic potential to the production of an approved drug involves pre-clinical testing, complex clinical trials in humans, and post-trial regulatory approval by the Food and Drug Administration (FDA). For drugs, this process can take 10 to 15 years and costs millions of dollars, with less than 12% of the potential drugs receiving final

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http://crosscurrentpublisher.com/ccijmb/

DOI : 10.36344/ccijmb.2020.v02i02.001

approval. Several factors, such as difficulties in harvesting organisms, low quantities of active compounds in extracts, finding adequate procedures for isolation and purification, possible toxicity of the compounds and sustainable production of compounds may slow down the entire pipeline.

Microalgae are eukaryotic plants that contribute up to 40% of global productivity (Moreno-Garrido, 2008). They are at the base of aquatic food webs, have short generation times (doubling time = 5–8 h for some species) and have colonized almost all biotopes, from temperate to extreme environments (e.g., cold environments and hydrothermal vents). Their advantage in marine drug discovery is their metabolic plasticity, which can trigger the production of several compounds with possible applications in various biotechnology sectors (i.e., food, energy, health, environment and biomaterials) (Lauritano *et al.,* 2016; Romano *et al.,* 2017). They can be easily cultivated in photo-bioreactors (e.g., in 100,000 L bioreactors) to obtain a huge biomass and represent a renewable and still poorly-explored resource for drug discovery. They use solar energy and fix CO_2 which contributes to the mitigation of greenhouse gas effects and the removal of nitrogen and phosphorous derivatives which can be pollutants depending on their concentration (de Morais *et al.,* 2015).

This study was conducted to evaluate the in vitro antiproliferative activity of methanolic extracts of *Chlorella vulgaris* and *Nostoc muscorum* against Human cervical Cancer cells (HeLa), liver hepatocellular *cells (*HepG-2), Human colorectal adenocarcinoma cells (HT-29) and Ehrlich Ascites Carcinoma Cells (EACC) in order to determine the bioacitivities of microalgae.

MATERIALS AND METHODS

Methanol extracts of *C. vulgaris* and *N. muscorum* were prepared based on the previously described methods. In brief, 10 g of dried algal samples was soaked in 1000 mL of 100% methanol at room temperature (21°C ± 1°C) for 20 min, followed by microwave assisted extraction using a microwave oven at power of 360 W, irradiation time of 7 s/2 min, and extraction time of 40 min. The extracts were then rapidly cooled to room temperature using an ice water bath and filtered through qualitative No. 1 filter papers. After that, the extracts were evaporated under reduced pressure (−10 mbar) at 40°C using a rotavapor and dried to constant weight using a freeze drier at −45°C, 0.1 atm for 48 h to achieve the extracts. The extracts were stored at −20°C until required for further experiments.

Cell Culture and Incubation of Tumor Cell Line

Cell line and culture HeLa cells were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Growth was maintained at 37°C in a humidified incubator containing 5% CO_2. Cells were within 13 passages when the experiment was conducted.

Human hepatocellular cancer cell line, HepG-2, was obtained from the American Type Culture Collection Centre (ATCC). Cells were maintained in RPMI-1640 supplemented with 100 µg/mL streptomycin, 100 units/mL penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere at 37 °C.

The human colonic adenocarcinoma cell line HT-29 was obtained from the American Type Culture Collection Centre (ATCC) and cultured in McCoy's 5A medium (PAA Laboratories, Pasching, Austria). The human melanoma cell line UACC-62 was cultured in RPMI medium supplemented with 2 mM L-Gln and 25 mM HEPES (Sigma, India). The culture media were supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cultures were incubated at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity Assay against HeLA and HT-29 cells

Microalgal extracts were tested for antiproliferative activity against HeLa and HT-29 cancer cells using the Sulforhodamine B (SRB) assay according to the previously described protocol (Skehan *et al.,* 1998). Briefly, cells (2.5 x 10^4 cells/mL) were incubated in 96-well plates with a serial dilution of extracts starting with 4,000µg/mL for 24, 48, and 72 h at 37°C with 5% CO_2. The cells were fixed with 50 µL of 50% cold trichloroacetic acid for 30 min at room temperature followed by gentle tap water washing (5x) and then drying. Cells were then stained with 100 µl of 0.4% SRB in 1% acetic acid for 30 min, followed by washing with 1% acetic acid (4x). The plate was dried, 100 µL of 10 mM Tris buffer were added to each well, and the plate was shaken for 5 min. Relative cell viability was measured by scanning at 540 nm on a microplate reader.

Cytotoxicity Assay against HepG-2

The cytotoxicity of crude extract was tested against HepG-2 by SRB assay as previously described (Skehan *et al.,* 1990). Exponentially growing cells were collected using 0.25% Trypsin-EDTA and plated in 96-well plates at 1000-2000 cells/well. Cells were exposed to test extract for 72 h and subsequently fixed with TCA (10%) for 1 h at 4 °C. After several washing, cells were exposed to 0.4% SRB solution for 10 min in dark place and subsequently washed with 1% glacial acetic acid. After drying overnight, Tris-HCl was used to dissolve the SRB-stained cells and color intensity was measured at 540 nm.

Cytotoxicity Assay against EACC

The viability percentage of tumor cells was measured after incubation with the tested algal extracts as well as DMSO as control. Two ml of cells (4×10^6 cells) were transferred to a set of tubes, then different algal extracts (200 µg/mL) were added to the propriate tubes as well as DMSO. The tubes were incubated at 37°C for 2 h. Then, in a test tube containing 80 µL saline and 10 µL trypan blue and 10 µL of cell suspension were added and mixed then the number of living cells was calculated using a hemocytometer (Bennett *et al.*, 1976).

Nitrogen Stress Experiments

The promising algal species; *Chlorella vulgaris* and *Nostoc muscorum* which recorded the greatest anticancer activities were cultured in culture media with increasing (1.5 (control), 3, 6, 9 g/L) or decreasing (1.5 (control), 0.75, 0.375, 0.0 g/L) nitrate nitrogen contents. Experiments were carried out in the previously used culture conditions of light intensity, temperature and experiment duration. Filtration of the culture media was followed by addition of distilled water, freezing, then anticancer activities were carried out on these extracts by the same methods used in the normal cultured algal species.

Anti-Proliferation Assay

The cytotoxicity was determined using the previously described formula (Bendale *et al.*, 2017): Cytotoxicity (%) = Optical density of (sample – blank)/ Optical density of (control – blank) X 100. The inhibition concentration of extract that is able to inhibit cell proliferation by 50% (IC50) was calculated graphically from the cell proliferation curve. The extract at the time point with the lowest IC_{50} value was selected for further analysis.

Morphological Analysis

Morphological changes of cancer cells treated with the selected extract were observed by phase contrast microscopy using a Zeiss Axio Observer Z1 (Carl Zeiss AG, Jena, Germany) and an Operetta high-content imaging system (Perkin Elmer) for fluorescent microscopy. The cells (2.5×10^4 cell/ mL, 0.1 mL/well) were incubated in a 96-well plate with 0.2 mL/well of three different doses (62.5, 125.0, and 250.0 µg/mL) of the selected extract for 48 h at 37 °C with 5% CO_2.

Flow cytometry and cell cycle determination

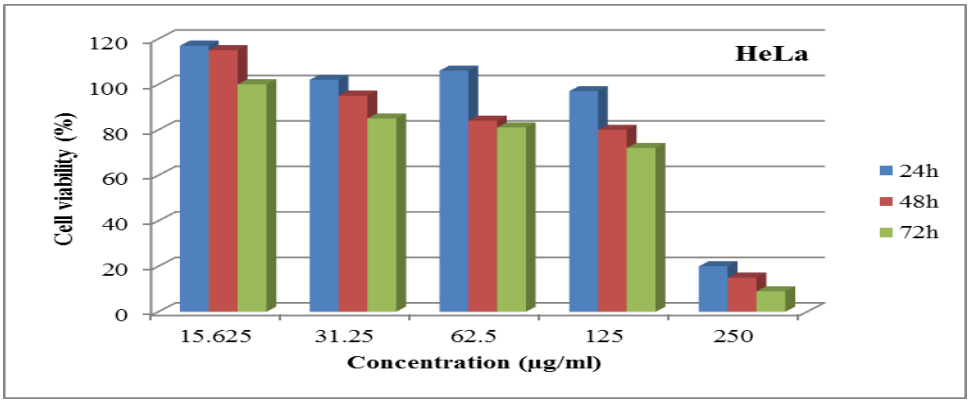
HT-29 and UACC62 cells were seeded at 8 x 10^5 cells/well in 6-wells NUNC plates and were treated with either MJ or OXLs for 24 h. Cells were treated with trypsin and collected by centrifugation (375 g, 5 min, 25°C), and fixed in 70% ethanol (10^6 cells ml^{-1}) for 24 h. Ethanol was eliminated and cells were resuspended in PBS containing 5 mg mL^{-1} pancreatic A ribonuclease (Sigma-Aldrich) and kept at 4°C for 48 h under mild agitation. Then, propidium iodide was added at 5 µg mL^{-1}, and cells were incubated at 4°C for 1 h. DNA content was determined on a Beckman Coulter Cytomics FC 500 MPL (Beckman Coulter Inc) with MXP Software examining 10^4 cells. Percentages of cells in apoptotic-sub G1, G0/G1, S and G2/M were calculated using CXP software.

Statistical Analysis

Data were subjected to an analysis of variance, and the means were compared using the "Least Significant Difference (LSD)" test at 0.05 and 0.01 levels, as recommended by Snedecor and Cochran (1982).

RESULTS AND DISCUSSION

Numerous factors are involved in cancer development, including genetics, health, nutrition, personal habits, and the environment. Cervical cancer is the fourth most common cancer among women globally; about 570,000 new cases were reported in 2018, representing 6.6% of all female cancers. Currently, the recommended treatment programme for this cancer involves surgery, chemotherapy, radiation, or other methods (World Health Organization, 2018). Herbal medicine has been increasingly recognized as a useful complementary and alternative therapy for patients with cancer. Numerous clinical studies have reported the positive effects of herbal medicines when taken with conventional therapeutics, in particular in terms of survival rate, the immune system, and quality of life of cancer patients (Yin *et al.*, 2013).



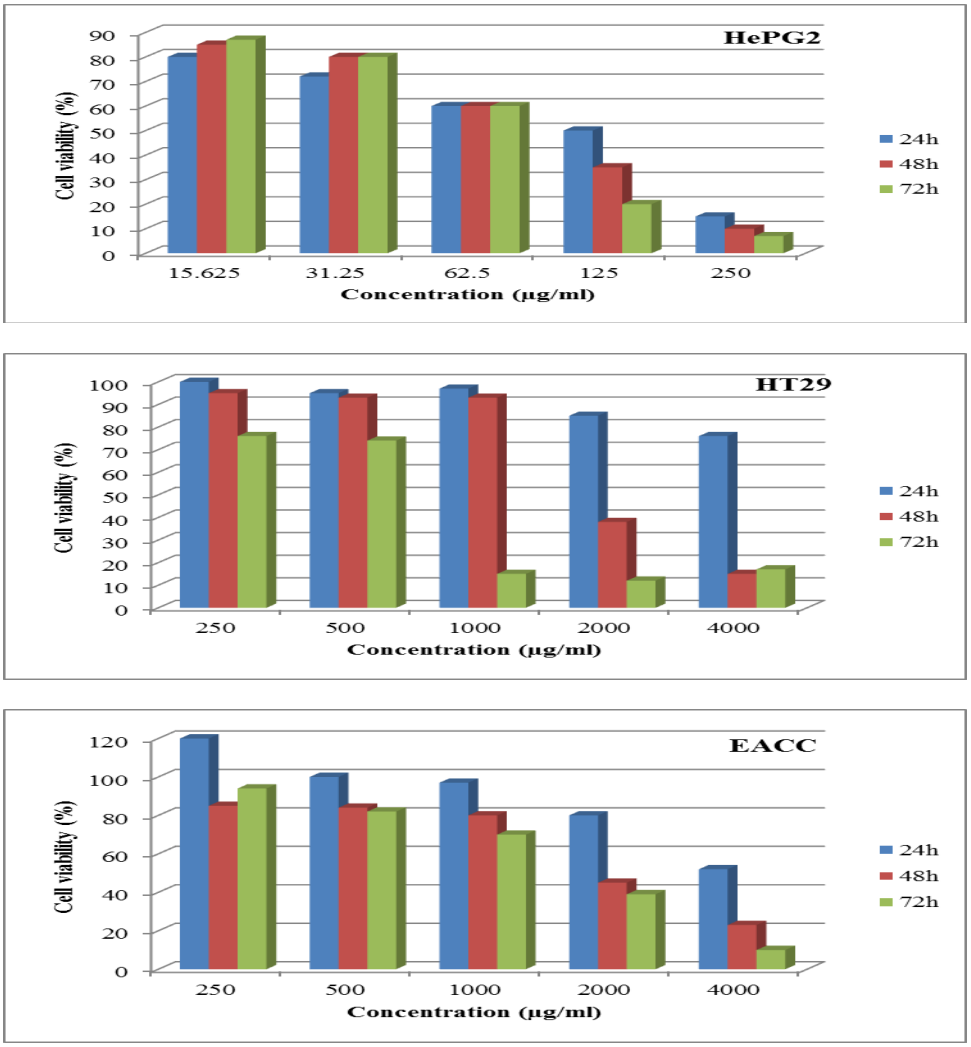


Fig-1: Cell Viability Assay. The cells were incubated with increasing concentrations of microalgal extracts for 24, 48, and 72 h, and cell viability was determined by the SRB assay. All values given are means ± SDs

Anticancer activity of the nitrate stressed promising algal species using EACC and HepG2 cell lines (%).

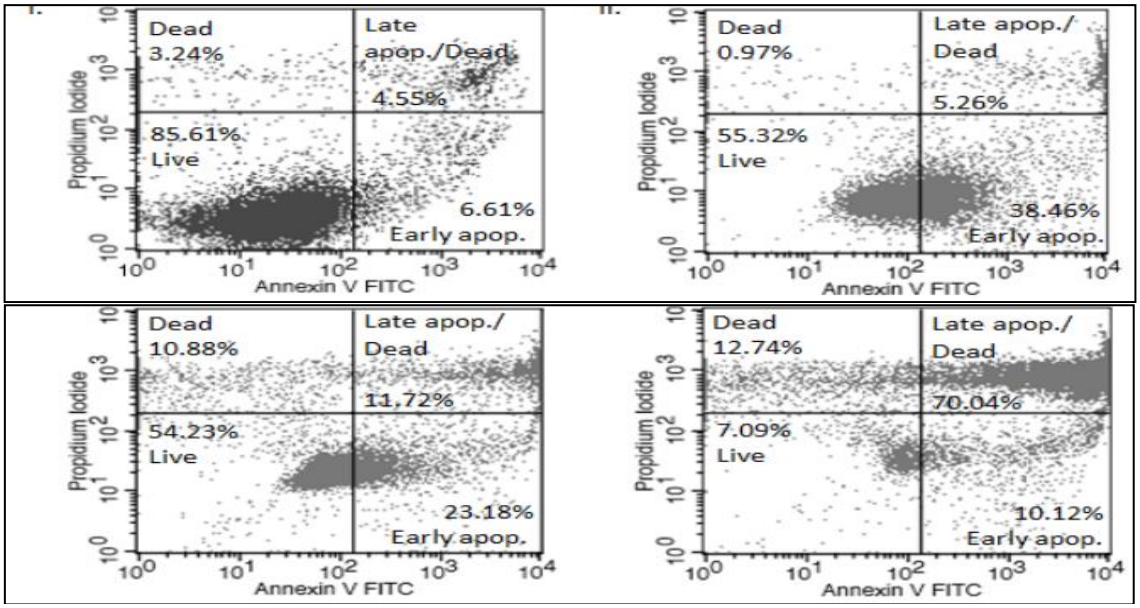
	N. muscorum	
Treatment	HepG2	EACC
Control	70.40	68.30
3 g/l	72.10	69.70
6	70.60	72.60
9	75.90	82.60
0.75	72.60	68.30
0.37	74.80	78.30
0.0	82.00	82.90
LSD	0.575	0.907

	C. vulgaris	
Treatment	HepG2	EACC
Control	88.60	85.90
3 g/l	85.60	84.60
6	86.90	85.60
9	88.70	90.40
0.75	86.70	83.00
0.37	88.00	84.00
0.0	92.30	89.90
LSD	1.63	0.505

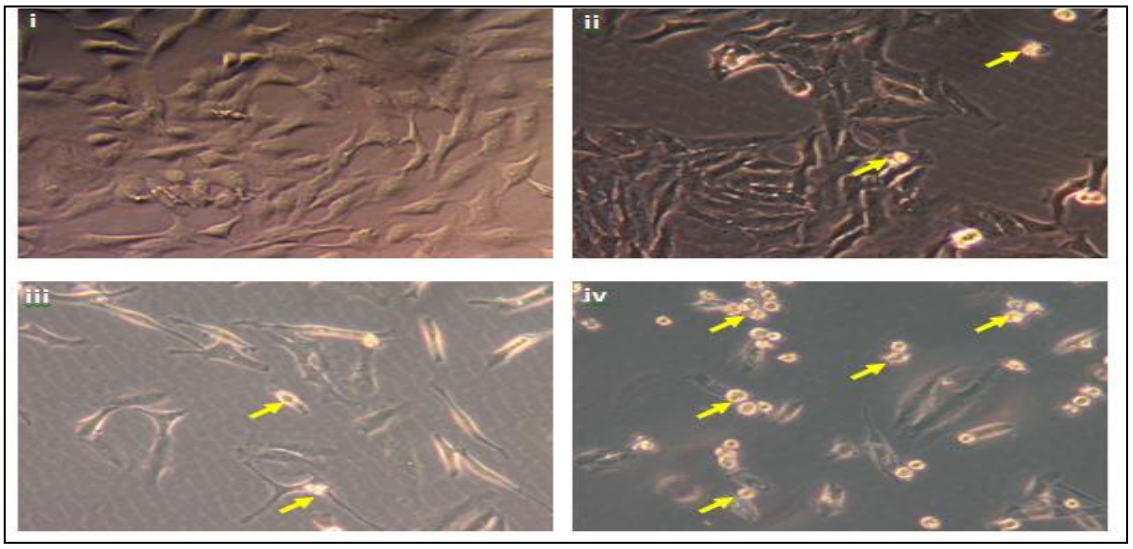
Each value is presented as mean of triplet treatments, LSD: Least different significantly at P≤0.05 according to Duncan's multiple range test.

Evaluation of apoptosis by flow cytometric analysis

After treatment of cancerous cells with microalgal extract for 48 h, 55% of the cells were viable, 38% were in early apoptosis, 5% were in late apoptosis, and 1% exhibited necrosis. Treatment with 125 µg/mL of the *C. vulgaris* extract increased the percentage of cells in late apoptosis (12%), and 70% of cells were in late apoptosis when treated with 250 µg/mL. The percentage of necrotic cells was less than 12% for all treatments.



Dot Plots (A) of untreated cells (i) and treated cells with 62.5, 125, and 250 µg/ ml of the microalgal extract, respectively, and the percentage (%) of cell distribution



Representative Images Showing Morphological Changes of cancer cell lines detected by Phase Contrast Microscope

Cell cycle analysis showed that the *C. vulgaris* extract induced S and G2/M phase arrest after 24 h of treatment and S-phase only after 48 h of treatment. DNA synthesis or duplication of chromosome occurs during S-phase, whereas the G2/M phase is a gap state (G2) in which the cell with newly replicated DNA continues to grow to ensure that everything is ready to enter the mitosis (M) phase. At the M stage, the cell stops growing and divides its copied DNA into two daughter cells. The observed S phase cell cycle arrest, which indicates decreasing rate of DNA synthesis, might explain the mechanism of the antiproliferative effect by the *C. vulgaris* extract at 48 h of treatment. In a previous study, the hexane fraction of CN increased the sub-G1 populations of CNE1, HepG2, and A549 cells in a dose-related manner, while the G0/G1 and G2/M populations showed a corresponding decrease (Ng *et al.*, 2017). Thus, the antiproliferative mechanism may differ depending on the type of cancer cell, the tested compound, and the different natural products present in the extract, which are likely to vary at different stages of the cell cycle (Peng *et al.*, 2015).

CONCLUSION

Further studies are needed to discover the mechanisms underlying the antiproliferative activity of microalgal extracts against cancer cell lines and to identify specific bioactive compounds that are responsible for the observed activities. Further, this information may be useful for the development of a novel anticancer therapy.

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