
Ali M. Najem¹, Ibrahim J. Abed¹, Laith Z. Fadhel¹

1. Department of Biology, College of science, University of Baghdad, Baghdad-Al-Jadiria, Iraq

*Corresponding author: laithzuhair2018@gmail.com (Fadhel)*

**Abstract**

Isolation of blue green alga *Spirulina platensis* was experimentally fulfilled. A pure isolate of the alga was extracted in soxhlet apparatus by a mixture of three different alcohols with three different polarities methanol, chloroform and hexane in a a ratio 2:1:2. Anticancer activity of the extract was evaluated against breast cancer which conducted by using five different concentrations (6.25, 12.5, 25.50,100 µg/ml) of *S. platensis* extract, after 72 hrs. O.D. readings showed highly cytotoxic impact against cancerous cells, this impact increased exponentially as the concentrations increased, and the Cytotoxicity test showed 50% inhibition (IC50) of cells at 19.18 µg/ml after 72 hours incubation for MCF7. In addition, a Real-time PCR study investigated the effect of *S. platensis* extract on P-53 gene, mutation in this gene is the common genetic change in human cancer, depending on the outcome data, results were remarkable showed stastical stimulation of the gene which coding for tumor cell suppressor protein.

**Keywords:** *Spirulina platensis*, P-53 gene, Breast Cancer Cells, extraction

**Introduction**

Adverse treatment had been projected diverse negative impacts during therapy events of patients which are involved in curing cancers, challenges in decreasing these treatments have been developed to manage their hazard effects on patients such as breast cancer including patients with metastasis even those who received inclusive therapy instead of poor outcomes. [1]. Genetic mutations are the main causes of cancers especially the inherited mutations beside many other causes such as diet, life style, smoking, metabolic mutations, pollutants (radiation), obesity and viruses. Those causative factors together or apart stimulate cancer [2,3]. Inhibition growth of cancers includes many steps the first one is chemotherapy in addition to a group of drugs, illness symptoms ranging from mild effect represented by noxious nausea, vomiting, baldness, mouth sores or canker sores and loss of appetite to life threatening effect, are the possible results of using such therapies. Therefore, new and revolutionary therapeutical protocols comprise from natural antitumor compounds derived from plants or algae should be urgently investigated. [4]. Such
investigations should be conducted due to immense impact of chemotherapy on both cancerous and normal cells according to their distinguished similarities, both cells share same DNA and main metabolic reactions. Thus, DNA replication and cell division of both normal and cancerous cells will be the affected severely resulting inevitably to highly toxic impact on one marrow and gastrointestinal system which could be fatal [5, 6]. [7]Elucidate treatment related mucosal injury and pathobiology occurs during treatment of cancer. Induction of cancer and protection from cancer both are the projection of the act of antioxidant and free radicals, free radicals produce by industrial solvents, stress, alcohol, heavy metals, pollution and transition metals are the stimulator of cancer, on the other side free radicals vicious role could be eliminated by antioxidant compounds produce by many metabolic pathways in vegetables and fruits which double the protection of human body against cancer due to their scavenging role[8].Biomedical experiments are targeting many biological activities such as antitumor, antiviral and immunosuppressant; these activities are displayed by microalgae including stimulation of the P53 gene responsible for suppression of tumour cells proliferation [9]. Bioactive metabolic compounds produce by algae especially are aimed due to their activities as antitumor. Dedication for the extraction of these compounds have been exponentially increased, such as betacarotene is a secondary metabolic compounds as tetraterpene is produced in high concentration in *Spirulina* which is a blue green alga, such compound inhibits formation of squamous cell carcinoma. [10]. Another compound isolated from *Spirulina platensis* called C-phycocyanin exhibited scavenging activity as antioxidant have been experimentally proved as anticancer [11].

**Materials and Methods**

**Isolation and purification of alga**

*S. platensis* isolated from Tigris river on longitude(44° 24' 4.9026”E) and latitude (33° 21' 56.2026”N) by collecting many samples of water in a sterilized glass containers, cultures of S. platensis was obtained by using serial dilution method included 1ml of sample inoculated into 9 ml of Chu-10 nutrient medium(HI media, Mumbai India). The procedure was repeated frequently with microscopic examination until eventually a pure isolate from *S. platensis* was obtained. The uni-algal isolate transferred to Chu-10 medium and incubated in the illuminated incubator with light intensity about 200 µE/m²/Sec and temperature was set 26± 1 C˚.

**Extraction of alga**

The uni algal liquid isolate was dried by adding drops from 0.1 N NaOH to precipitate the alga in order to facilitate discarding most of the liquid medium, then the rest of the medium poured in clean sterile petri dishes and evaporated in oven with temperature did not exceed 50 C˚. Algal mass remained in the dishes washed with distilled water and dried by the same procedure. Three different polarity of methanol, chloroform and hexane in a ratio 2:1:2 was blend to gather and mixed with the dried algal powder in a ratio 1:20 and this extraction procedure accomplished by using soxhlet apparatus with continuous extraction for at least 8 hrs according to [12].
Maintenance of cell cultures

RPMI-1640 and 10% Fetal bovine serum (Capricorn, Germany) were used to maintain MCF-7 cells, in addition to Penicillin (100 units/mL) and streptomycin (µg/mL). Cells were passaged by using Trypsin-EDTA (Capricorn, Germany) and reseeded at 80% confluence twice a week, and incubated at 37 °C[13].

Cytotoxicity assay of the extract

To determine the cytotoxic effect of S. platensis extract, the MTT cell viability assay was done using 96-well plates. Cell lines were seeded at 1 × 10^4 cells/well. After 24 hrs, cytotoxicity test was applied by treating cells with five different concentrations (6.25, 12.5, 25, 50, 100 µg/mL) of the algal extract, after 72 hrs. medium was removed in order to measure cell viability. The procedure proceeded by adding 28 µL of MTT solution (2 mg/mL) (Bio-World, USA) then at 37 °C, cells were incubated for 2.5 hrs. MTT solution removed, the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) (Santacruz Biotechnology, USA) followed by 37 °C incubation for 15 min with shaking [14]. At 492 nm the absorbency was assayed on a microplate reader (Gennex Lab, USA). The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:-

\[
\text{Cytotoxicity} = \frac{A - B}{A} \times 100
\]

A and B are the optical density of control and the optical density of test.

Microscopic examination of treated MCF-7 cells

For visualize the shape of cells under inverted microscope, 96-well micro-titration plates were seeded with 200 µL of suspensions of cells at a density 1x10^4 cells/mL were seeded. After 48 hrs of incubation at 37°C, the algal extract was applied after the medium was removed. 24 hrs. later, 50 µL of Crystal violet used to stain the plates and then incubated for 15 min at 37°C. The stain was washed gently with tap water until the dye was removed. The cell observed under inverted microscope at 100x magnification microscope filmed and photographed with digital camera [15].

Real-time PCR

Modifications in hippocampal expression genes were investigated in the aid of PCR. Depending on NCBI database, sequences for the primers were designed which included:

\[
\text{P53 (forward:} 5′-\text{CCGTCCCAAGCAATGGATG-3′)}
\]
\[
\text{ (reverse:} 5′-\text{GAAGATGACAGGGGCCAGGAG-3′)}
\]

Each RT-PCR reaction combination containing 7.5 µL SYBR green, 1 µL of cDNA, zero.3 µL related primers, zero.3 µLRox, and the final volume was completed up to 15µL with 5.6 µL of distilled water.
SYBR Premix Ex. Taq™ kit used for the assay. In a react to double-stranded DNAs, the emission intensity of SYBR green detected by PCR and that achieved by the implemented Biosystems (ABI) Prism Sequence Detection system. GAPDH mRNA had been used as an inner control to identify the relative expression amount of the genes. The equation below is used to calculate the value of Threshold Cycle (CT) where the Reporter fluorescence is greater than the brightness at the Threshold which represents the amount of gene expression of isolates under study as follows (refer):

\[ \text{The fold change} = 2^{\Delta \Delta CT}, \quad \Delta \Delta CT = \Delta CT \text{ (treated target gene)} - \Delta CT \text{ (treated R16)} - \Delta CT \text{ (untreated target gene)} - \Delta CT \text{ (untreated R16)} \] [16].

**Statistical analysis**

The obtained data were statically analyzed by using an unpaired t-test with GraphPad Prism 6. The values were presented as the mean ± SEM of triplicate measurements [15].

**Results**

Five different concentrations (6.25, 12.5, 25.50, 100 µg/ml) of alcoholic extract of *S. platensis* which where extracted by using three different solvents with different polarities to exploit a broad range of compounds. These phytoactive compounds represented by the algal extract were tested as potential inhibitory curative drug and anti-tumor agent against human breast cancer cell line (MCF7). Cytotoxic activity of the extract against MCF7 cell line was tested and estimated after 72 hrs of application, and that carried out in a comparison to cell control. Cytotoxicity test showed 50% inhibition (IC50) of cells at 19.18 µg/ml after 72 hours incubation for MCF7, as a response to the dose which considers a low concentration such as 19.18 µg/ml but cause high inhibition reached to 50% inhibition (IC50) of cells. Remarkable results exhibited distinctive cytotoxic effects of *S. platensis* extract, according to O.D. readings of the cell viability, the percentage of tumour cell inhibition or the percentage of cytotoxicity exponentially increased as the dose or the concentrations increased in a regular pattern as shown in figure (1) and proved by making microscopic slides showed the distinctive curative influence on cancer cells treated with *S. platensis* extract as appeared in slide (A) in a comparison to control or untreated cancer cells in slide (B) in figure (2).
These results along with similar few studies paving the way for revolutionary curative and pharmacognostic solution for the most notorious dilemma of the last and current millennium depending on phyto therapy especially algae. The outcome data of [17] showed high resemblance to that resulted from this study and both studies manifested inhibitory impact against human breast cancer cell line by using crude extract of *S. platensis*. [18] Reported that *Spirulina* extracted by methanol treated Ehrlich Ascites Carcinoma (EAC). The filamentous blue green alga *Spirulina* metabolism pathways produce vast primary and secondary metabolites with variable nutritional and curative values as a result to the high concentrations of these compounds, researches showed that *Spirulina* extracts multiply phagocytic effect of macrophages and induced production both antibodies and cytokines. In addition to that, Studies spotlighted the important metabolic booster role of *Spirulina* in many pathways such as lipids and carbohydrates. Diverse therapeutic roles of *Spirulina* were documented against cancer and HIV [19,20].
Induction of P-53 gene

Figure: Expression of p53 gene in MCF-7 after treated with S. platensis at concentration 19.18 µg/ml

Gene alteration of cell line was investigated using Real-time quantitative PCR. In this experiment, 2 main type of gene were measured to identify the pathway of apoptosis and the mechanism action. These genes include P53, and Caspase-8. This gene coding for tumor cell suppressor protein, which regulates cell proliferation, that damage in DNA increase level of this gene leads to apoptosis, mutation in P-53 gene is the common genetic change in human cancer [21]. As illustrate in figure (3), results showed the remarkable impact of S. platensis extract in stimulation of P-53 gene expression enormously in comparing to the control or untreated cells. Phycocyanin is main photosynthetic pigment produce abundantly by blue green algae Spirulina, [22,23] demonstrated the major role of this pigment isolated from spirulina in induction of apoptosis of MCF7 cells by regulation of NF-κB, P53 and Bcl-2 expression.

Conclusion

Results fulfilled the purpose for which this study was conducted, high inhibitory effect of S. platensis alcoholic extract against breast cancer cells in five different concentrations and remarkable stimulation of p-53 gene by increasing the expression level of this gene after being completely inactivated and that achieved by the alcoholic extract contains compounds derived from the miraculous S. platensis.
References


