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Advances in Clinical Toxicology
ISSN: 2577-4328

Spirulina Polypeptides Inhibit the Growth of Human Lung Tumor (H460) Cells

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Research Article
Volume 10 Issue 1
Received Date: September 17, 2024
Published Date: February 06, 2025
DOI: 10.23880/act-16000326

Abstract

Blue-green algae, or cyanobacteria, are some of the oldest organisms on Earth which grow even in extreme conditions. These algae have biologically active compounds which may contribute to the treatment of cancer due to their inhibitory effect against cell cycle and proliferation. Biologically active polypeptides obtained from the filamentous cyanobacterium called *spirulina* have considerable amounts of proteins and medicinal properties. *Spirulina* is shown to have benefits across a range of human health indications from malnutrition to anticancer properties. In this research, polypeptides were hydrolyzed from proteins present in *spirulina* and their anticancer effects were studied using assays such as MTT, Live/Dead, and Cell proliferation in Human Lung Tumor (H460) Cell model.

Keywords: Blue-Green Algae; Cyanobacteria; *Spirulina* Polypeptides; Anticancer

Abbreviations

DMEM: Dulbecco's Modified Eagle Medium; RPMI: Roswell Park Memorial Institute; FBS: Fetal Bovine Serum.

Introduction

Different kinds of microalgae have been found to be the source of number of bioactive compounds having amino acid fragments with the efficiency of various biological applications [1]. Increased levels of protein content in these microalgae have been found to be the reason for their use in various potential applications [2]. Abundantly available in nature, microalgae are photosynthetic organisms used widely as a non-toxic and biocompatible alternative source in potential medical applications such as wound healing, tissue engineering, antitumor activity, and various other therapeutic applications [3]. These organisms are found in environmental sources such as oceans, soils, and various other ecofriendly sources [4]. *Spirulina* polypeptides are studied for the efficiency depending on their antibacterial, antitumor, antiallergic, and antihypertensive properties [5]. *Spirulina*, filamentous blue green algae have acquired considerable importance in fields such as pharmacology, food, and agriculture, because of their constitution of proteins, vitamins, minerals, and amino acids [6]. *Spirulina* has essential effects towards oxidative stress based on the presence of an enzyme called superoxide dismutase [7]. Based on these properties, the extracts from *spirulina* have been reported to be efficient in inhibiting the growth of various human cancer cells [8]. The evidence of effectiveness of *spirulina* in cancer is extremely limited as far as the clinical trials are concerned. The *spirulina* studies conducted on various types of carcinogenesis show a degree of similitude but are in a haphazard state [9]. S. platensis and S. platensis-derived tetrapyrroles were experimentally tested for

pancreatic cancer and it was reported that it substantially decreased the proliferation of experimental pancreatic cancer [10]. Arthrospira platensis extract showed a remarkable effect on the lung cancer cell cycle and prevented the proliferation of cancer cells, and it could protect the normal cells by its antioxidant activity [11].

Therefore, there is a need for extensive research to explore novel antioxidant molecules from algae, and their purification strategies. Studies are needed to explore the actual antioxidant compounds present in aqueous extracts of algae that have shown anticancer activities, and to investigate their mechanism of action on the cellular system and their capability to potentiates chemotherapeutic drugs [12].

The current study applied flash hydrolysis process to selectively hydrolyze proteins from *Spirulina* in the form of water-soluble peptides. Flash hydrolysis is a subcritical water-based continuous process with a residence time in the range of 10-12s [13-21]. It has been frequently used to hydrolyze proteins from different algae species [13,19,21-26]. The process has been shown to hydrolyze upto 2/3rd of algae proteins in water-soluble peptides form having molecular weight distribution ranging between 200 and 800 mass units (m/z) [15]. The water-soluble peptides produced from the flash hydrolysis of *Spirulina* slurry were freeze-dried and the recovered powder was applied to study its anticancer effects.

Experimental

Flash Hydrolysis

2 liters of 1% *Spirulina* (Arthrospira platensis) slurry was prepared using deionized water. The slurry was continuously stirred at 350 rpm for 1 hour using a magnetic stirrer to mix the slurry homogeneously. The final prepared slurry was transferred to a container to perform flash hydrolysis. During the flash hydrolysis process, an electric stirrer was used to continuously mix the slurry to provide a constant input. The slurry was fed into the flash hydrolysis system shown in Figure 1 using a 0.5 HP pump (Lewa pump). The flash hydrolysis system is equipped with a chiller (Pfannenberg), induction heating and a PID controller, (GH Induction Atmospheres), back pressure regulator (Equilibar), thermocouple (Omega), and a stainless-steel tubular reactor (0.79 cm internal diameter, and 40.6 cm long). The flow rate of the *Spirulina* slurry into the system was adjusted using the pump. A chiller system with 20% glycol mixture as a coolant was used to keep the biomass in a liquid state and keep the system in a set temperature. The system is equipped with an induction heating system of 5 kW with maximum power of 15 kVA at 230V. It is connected to a PID which was used to set the temperature of the reactor, define the heating rate, and monitor the temperature inside the reactor. A thermocouple was inserted below the reactor to measure the real-time temperature of the reactor. Additionally, the pressure within the system was maintained using a back pressure regulator.

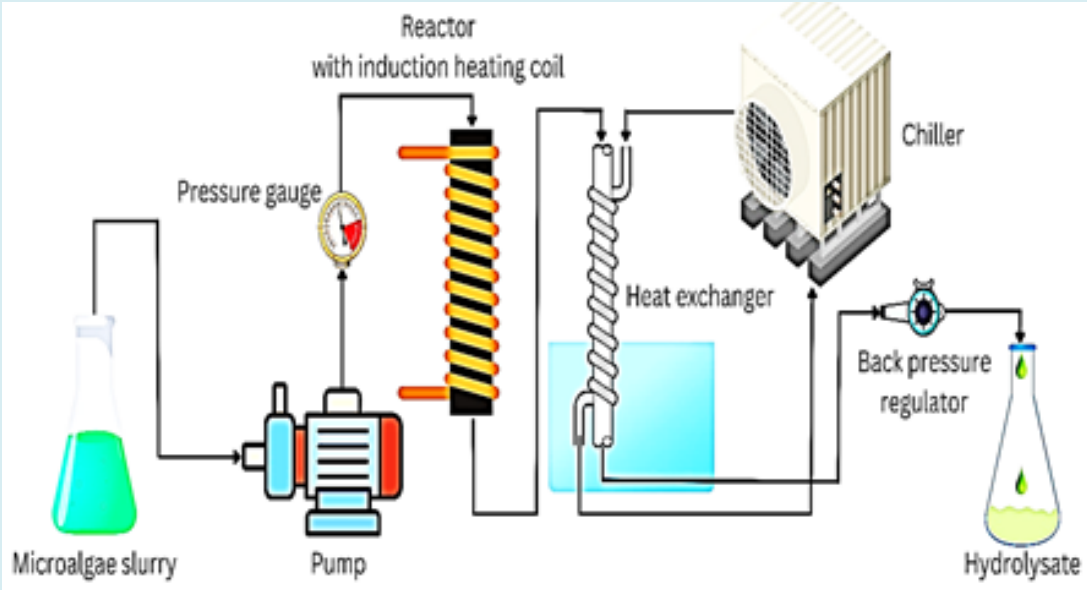

Figure 1: Schematics of flash hydrolysis system.

The residence time was calculated using Equation 1 where V is the volume of the reactor (mL), F is the flow rate of the slurry (mL s^{-1}), ρ_{pump} is the density of water at pump conditions (g mL^{-1}), $\rho_{p,T}$ is the density of water at specific pressure and temperature (g mL^{-1}).

$$t = \frac{V}{F\left(\frac{\rho_{pump}}{\rho_{p,T}}\right)} \quad (1)$$

First, the chiller was operated for 15 minutes to reduce the temperature of the system. The pressure of the system was set to 1550 ± 25 psi using compressed air and back pressure regulator. The system was then heated to 185°C using the induction heating system. The 1 wt% *Spirulina* slurry was then fed into the system at a flow rate of 92 mL min-1 with a residence time of 11 ± 2 s. After achieving a constant flow at the outlet, the liquid hydrolysate exiting from the system was collected. The hydrolysate was vacuum filtered, and the remaining liquids were centrifuged. A freeze dryer (Labconco) was used to freeze-dry the liquid hydrolysate. The freeze-drying process was carried out at about -54°C and 0.608 mBar pressure. The freeze-dried sample was placed in a sealed container and used for studying its anticancer effect.

Analyses of Freeze-Dried Hydrolysate

Elemental composition (Table 1) of raw *Spirulina* and freeze-dried *Spirulina* hydrolysate was determined by Flash 2000 Elemental Analyzer by Thermo Scientific using helium as a reference gas and oxygen as career gas. Approximately 1 mg of the sample was placed in a tin capsule with dimension of 3.3mm × 5mm for combustion at 950 °C. The elemental analysis was done in duplicate and the reported values are the average of the two values with a standard deviation of less than 3%.

Cell Culture

The human lung epithelial (Beas-2B) cells, and human lung tumor (H460) cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) media supplemented with fetal bovine serum (FBS), and penicillin streptomycin and the cells were stored in the incubator maintained at the temperature of 37°C and 5% CO_2. The Beas-2B and H460 cells were sub cultured, and the cells were counted using a hemocytometer to determine the number of cells to be plated for the biocompatibility assays such as MTT, and Live/Dead, and the cell proliferation assay was performed to confirm the biocompatibility assay results of H460 cells.

MTT Assay

A 96-well microtiter plate was used in this analysis and 5000 cells were seeded in each well of the plate. The plate was then placed in the incubator maintained at 37°C and 5% CO_2 for 48 hours. After this incubation, the cells were washed with DPBS and treated with lower to higher concentrations of *spirulina* polypeptides and incubated for another 48 hours. The stock solution of *spirulina* polypeptides was prepared by adding *spirulina* polypeptides to RPMI medium in the ratio of 1:1. After the second incubation, the cells were washed with DPBS and treated with the MTT dye and incubated for 3 hours. The MTT dye was prepared by adding them to DPBS in the ratio of 5:1.

Live/Dead Assay

A 6-well microtiter plate was used for this analysis and 250,000 cells were plated in all the wells. After plating the cells, the plate was incubated for 48 hours at 37°C and 5% CO_2. After 48 hours, the cells were washed with DPBS and treated with different concentrations of *spirulina* polypeptides and incubated again for 48 hours. After this incubation, the cells were treated with the dye mixture prepared by mixing the live and dead components of the dye. This step was carried out in the absence of light and the plate was covered with aluminum foil and placed at room temperature for 30 minutes.

Cell Proliferation Assay

On the first day, 250,000 cells were plated in a 6-well microtiter plate and incubated for 48 hours at 37°C and 5% CO_2. After the first incubation, the cells were treated with various concentrations of *spirulina* polypeptides after washing with DPBS. The plate was then incubated for 48 hours. After this incubation, the cells were treated with the detection reagent containing three components such as nucleic acid stain, background suppressor, and RPMI media. The plate was then incubated for 30 minutes.

Results and Discussion

Elemental Analysis

In Table 1, it is shown that the nitrogen, carbon and hydrogen percentage in the freeze-dried *spirulina* hydrolysate has decreased in comparison to raw *Spirulina* which is because of the distribution of it in the solid fraction that is composed of 1.99 % nitrogen, 10% carbon and 1.41% hydrogen. However, the presence of significant amount of these elements in the hydrolysate shows that the algae protein has been hydrolyzed to water-soluble peptides as reported in Kumar S, et al [15].

Name	Nitrogen %	SD	Carbon %	SD	Hydrogen %	SD
Raw *Spirulina*	9.85	0.6	43.39	2.7	6.14	0.3
Spirulina hydrolysate	9.43	0.4	37.48	1.3	5.58	0.2

Table 1: Elemental analysis of *Spirulina*.

MTT Assay

During the 3-hour incubation, the metabolically active Beas-2B and H460 cells absorbed the MTT dye and got converted into insoluble formazan crystals. These formazan crystals were dissolved by adding DMSO after removing the media. The microtiter plate was then placed on a rocker for 20 minutes for properly dissolving the formazan crystals. The absorption of MTT dye by the cells was measured by analyzing the plate using a spectrophotometer at the wavelength of 570 nm. The absorption values were recorded, and a plot was created with cell viability against the concentration of *spirulina* polypeptides and the results are indicated in the Figures 2 & 3. The results indicated that the viability of Beas-2B cells did not vary much with the *spirulina* polypeptide concentration whereas the H460 cell viability decreased with dosage-based concentration of *spirulina* polypeptides, when compared to the control (cells treated with no polypeptides). Significant decrease in the viability of H460 cells was identified from 5µg/µL to 100 µg/µL concentrations of *spirulina* polypeptides.

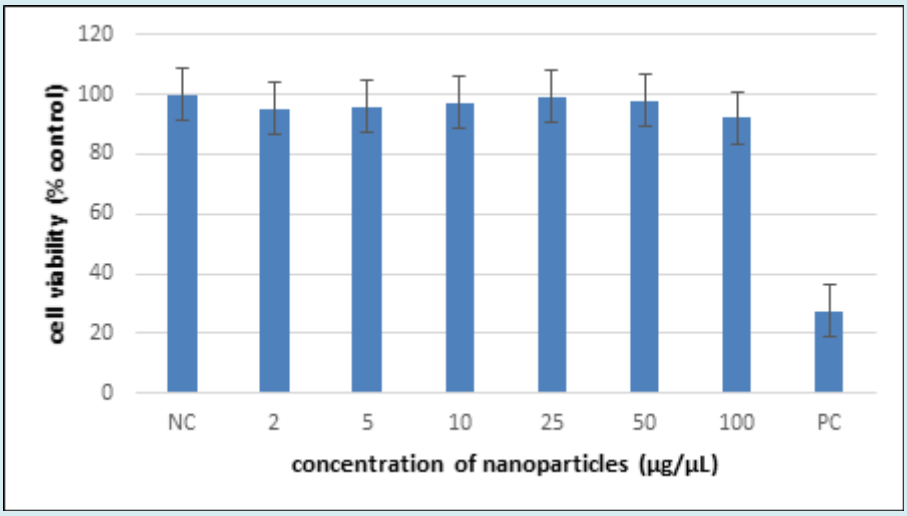

Figure 2: MTT assay representing the viability of Beas-2B cells treated with *spirulina* polypeptides.

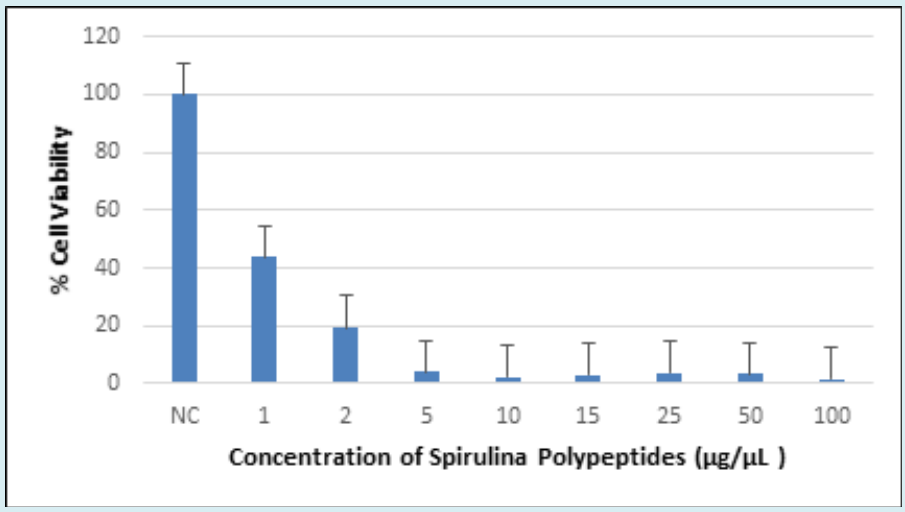

Figure 3: MTT assay representing the viability of H460 cells treated with *spirulina* polypeptides.

Live/Dead Assay

The MTT assay results were further confirmed by the live/dead assay. In the 30-minutes incubation, the metabolically active cells take up the green component of the dye and the dead cells take up the red component of the dye. After the incubation, the media was removed and DPBS was added to the cells and the fluorescence was analyzed using

the fluorescence microscope with the FITC filters for live cells and TRITC filters for dead cells. The Live/Dead assay results are shown in Figures 4 & 5. The results of this assay supported the results of MTT assay thereby indicating the anticancer activity of *spirulina* polypeptides towards the H460 cells based on their metabolic activity.

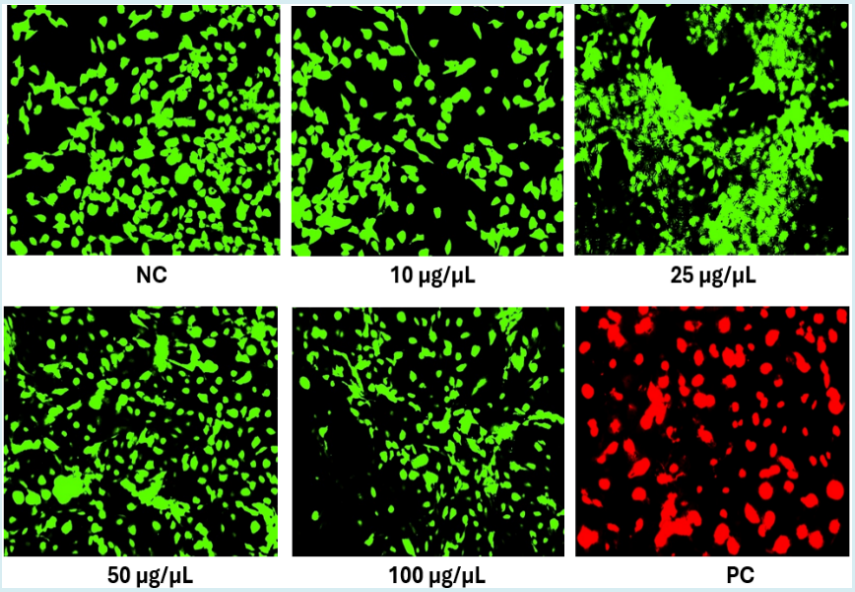

Figure 4: Live/Dead assay representing the viability of H460 cells treated with *spirulina* polypeptides.

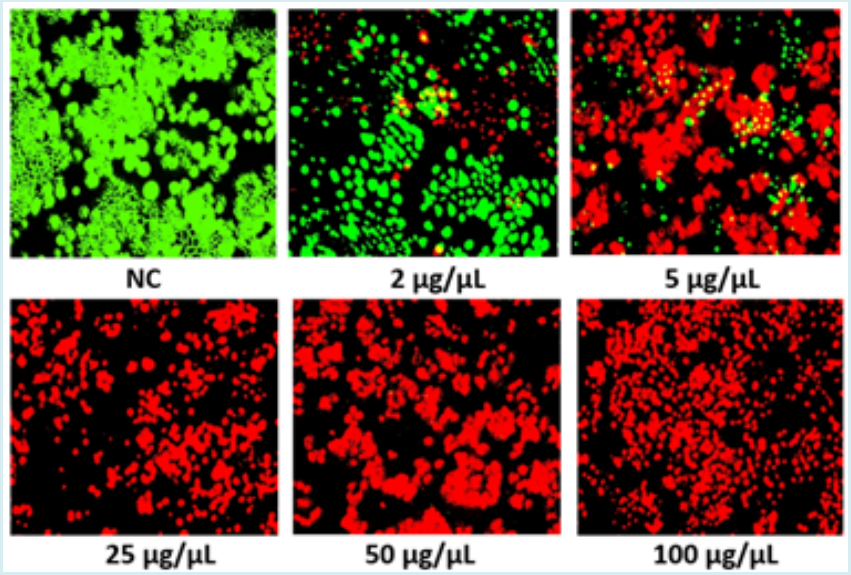

Figure 5: Live/Dead assay representing the viability of H460 cells treated with *spirulina* polypeptides.

Cell Proliferation Assay

Over the period of the cell proliferation assay, the cells multiply and the population of cells after treatment with *spirulina* polypeptides was analyzed based on the DNA content of the cells. In this assay, the reagent stains only the healthy cells after cell division. The background suppressor in the reagent suppresses the background fluorescence to obtain better image quality. The results in Figure 6 indicate that the population of cells decreased with the concentration of *spirulina* polypeptides and significant decrease was identified for the cells treated with 5µg/µL of *spirulina* polypeptides and further concentrations. This assay determined the anticancer activity of *spirulina* polypeptides towards the H460 cells based on their DNA content and assured the results of MTT and Live/Dead assays.

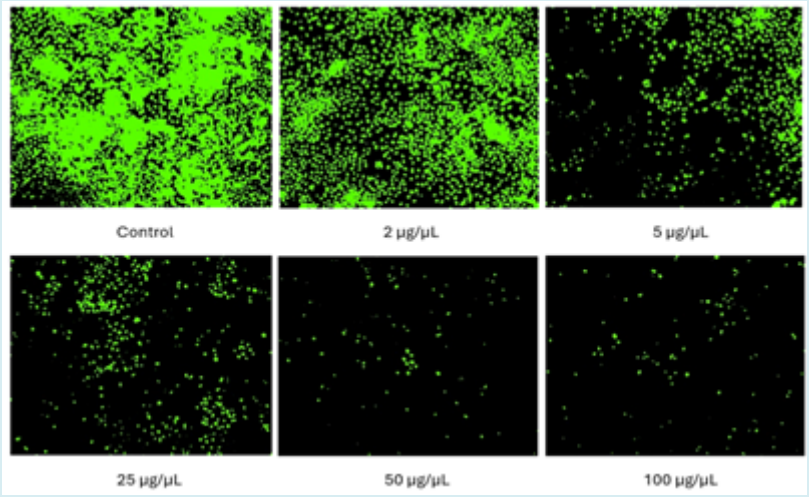

Figure 6: Cell proliferation assay representing the multiplication of healthy H460 cells treated with spirulina polypeptides.

Conclusion

The polypeptides hydrolyzed from *spirulina* plantesis were analyzed using biocompatibility assays towards the human lung epithelial (Beas-2B) cells and human lung cancer (H460) cells. The MTT and Live/Dead assays determined that H460 cells showed anticancer activity towards the *spirulina* polypeptides based on the metabolic activity of the cells that was further assured by the cell proliferation assay based on the DNA content of the cells.

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