

Anticancer and Antibacterial Properties in Peptide Fractions from Hydrolyzed *Spirulina* Protein

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ABSTRACT

Spirulina platensis is an edible microalga with high protein content (60-70%). Presently, there is a rising interest to evaluate *in vitro* cytotoxic effect of edible protein after hydrolysis by the gastric protease. Unfortunately, despite widespread researches about the health effect of hydrolyzed proteins in dairy products, very few studies are available in the field of marine microalgae protein. Therefore, this research was aimed to investigate anticancer and antibacterial effects of the dominant protein of *S. platensis* after hydrolyzed by Trypsin and Chymotrypsin enzymes on Human colon adenocarcinoma cell and *Escherichia coli*, and *Staphylococcus aureus*, respectively. The results revealed that ~ 20-22 kDa protein and its derived peptides decrease bacterial growth and <3kDa peptide fraction was able to significantly reduced SW480 cell viability. Based on this study, we can conclude that *Spirulina platensis* is a potential protein source in the future industrial production of functional peptides.

Keywords: Aanti-microbial effect, Chymotrypsin hydrolyzed peptides, *Spirulina platensis*, Trypsin.

INTRODUCTION

In recent years, reports demonstrate a correlation between diet and chronic diseases, consequently potential prevention of food were studied against chronic disease such as cancer, atherosclerosis, and osteoarthritis (Freitas et al. 2012). In this respect, nutraceuticals are nutrients from food or food products which are used as supplement diet and facilitate the prevention or treatment of a disease and/or disorder (Kalra 2003).

Sources of nutraceutical ingredients exist in many different reservoirs including the terrestrial and marine environments. Our knowledge about functional ingredients from terrestrial environment is far more explored than the marine environment (Simoons 1990). The marine environment has different characteristics such as various degrees of salinity, temperature, pressure, and illumination instructs particular interest in

compounds derived from marine organisms. These conditions increase the availability and chemical diversity of marine nutraceutical ingredients (Freitas et al. 2012). Intarasirisawat et al. (2012) reported antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonus pelamis*) roe, hydrolyzed by Alcalase 2.4 L (RPH) and the results revealed that it could be used as food additives (Intarasirisawat et al. 2012). Also, antiproliferative activity of 18 fish protein hydrolysates was measured on 2 human breast cancer cell lines grown *in vitro* and their preliminary data suggest that fish protein hydrolysate could represent an interesting source of anticancer peptides or lipids to be explored (Picot et al. 2006). In another study, Roe protein hydrolysates were reported to have anticancer effects and induced oxidative stress in Ca9-22 cells in terms of Reactive Oxygen Species (ROS)/superoxide generations and mitochondrial depolarization.

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Taken together, these data suggest that URH is a potential natural product for anti-oral cancer therapy (Yang *et al.* 2016).

Scientific reports that show algae, as marine organism, have many compounds that are present in nutraceuticals. Microalgae, as the main group of edible algae, have several prevalent types in food supplements and nutraceuticals such as: *Nostoc*, *Botryococcus*, *Anabaena*, *Chlamydomonas*, *Scenedesmus*, *Synechococcus*, *Parietochloris*, and *Porphyridium*, etc. The capability of these microorganisms are in producing necessary vitamins including: A (Retinol), B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B6 (Pyridoxine), B9 (Folic acid), B12 (Cobalamin), C (L-Ascorbic acid), D, E (Tocopherol), and H (Biotin). Edible microalgae concentrate essential elements such as potassium, zinc, iodine, selenium, iron, manganese, copper, phosphorus, sodium, nitrogen, magnesium, cobalt, molybdenum, sulfur, and calcium. Essential amino acids, omega 3, and omega 6 as other nutraceutical compounds are produced by microalgae (Vonshak 1997; Sedighi *et al.* 2016, Darvish *et al.* 2017).

Spirulina is a prokaryotic cyanobacterium that has been commercially produced since thirty years. Most usages of *Spirulina* are fish food, vitamin supplements, food dyes, aquaculture, pharmaceuticals, and nutraceuticals (Belay *et al.* 1993).

Spirulina has 60-70% protein by weight and many amino acids consist of the proteins (Becker *et al.* 1986). Some health effects of *Spirulina* are listed in scientific reports like weight loss, diabetes, high blood pressure reduction, and hypertension, antiviral and anticancer properties (Takai *et al.* 1991; Iwata *et al.* 1990; Hernández-Corona *et al.* 2002; Tang & Suter 2011; Schwartz *et al.* 1988; Mishima *et al.* 1998; Simopoulos 1991). Decaire *et al.* (1995) showed the positive effect of *Spirulina* on HDL increasing level and reduction in cardiovascular diseases risk (Decaire *et al.* 1995). Also, C-phycocyanin of *Spirulina* has antioxidant and anti-inflammatory properties (Romay *et al.* 1998). Furthermore, it has been shown that

Lactobacillus population increased after amendments of rat diet by *Spirulina* and absorption and digestion of food could improve when it was used in human diet (Tsuchihashi *et al.* 1987).

As mentioned above, *Spirulina* is a good source as a protein supplement. Theoretically, peptides are released from proteins food during the digestion process in the gut and can thus affect downstream health and cellular functions. To our knowledge, no report is available dealing with functional properties of *Spirulina* proteins.

The objective of this research was, therefore, to optimize intracellular dominant proteins extraction of *S. platensis* and then to evaluate the *in vitro* cytotoxic effects of their produced fractions from gastric protease hydrolysis against pathogenic bacteria and colon cancer cell line.

MATERIALS AND METHODS

All used chemicals were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Trypsin (16.730 unit/vial activity) and Chymotrypsin (68.9 unit mg⁻¹ activity) were purchased from Worthington Biochemical Co. (Lakewood, New Jersey). All solutions, prepared with double-distilled water, were kept at 4°C before further use.

Microorganism, Cultivation Conditions, and Biomass Processing

The microalgae *S. platensis* was obtained from the Persian Gulf and kept at 4°C prior to use. It was initially cultivated at Zarrouk's medium by adapting the procedure reported elsewhere (Zarrouk 1966). Briefly, the inoculated flasks were cultured at 32°C under manual shaking and irradiated at 6,000 Lux. The growth rate was recorded in consecutive days past-culture using the UV-visible spectrophotometer and ELISA reader. *S. platensis* cells were centrifuged at mid-log

growth phase at 1,500×g for 15 minutes and stored as pellets at -20°C.

Protein Extraction and Purification

Aliquot of 1 gr frozen *S. platensis* was washed in distilled water and suspended in 5 mL preparation buffer (50 mM Tris, 10 mM magnesium chloride, 20 mM Potassium chloride, 1 mM PMSF). The mixture was kept at room temperature for 30 minutes and centrifuged at 6,000×g. Then, cell walls were broken using liquid nitrogen for 30 minutes and cell debris was re-suspended in preparation buffer and centrifuged at 10,000×g for 1 hour. The supernatant was concentrated with 4M ammonium sulfate for 24 hours and the protein precipitate was dissolved in the buffer including 10 mM Tris-HCL, 50 mM NaCl, 1 mM Na₂EDTA.

Protein content determination was carried out using the Bradford method (Bradford 1976), which consists of a spectrophotometric determination at 595 nm, using Comassie Blue G-250 as a protein binding dye. Bovine Serum Albumin (BSA) was used as standard protein.

Separation of the proteins based on molecular weight was carried out using Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, CA, USA). According to the instructions of the manufacturer, samples with ratio of 5:1 were mixed with SDS sample buffer and boiled for 5 minutes in 95°C. Gels with 10% concentration were stained with silver nitrate.

Size Exclusion FPLC Analysis

The protein samples were filtered through a 0.22 µm filtration membrane (Millipore). An aliquot of 500 µL of obtained protein was injected manually on Superdex 75 10/100 GL size exclusion column using 50 mM sodium acetate and 150 mM sodium chloride (pH 4.8). Analysis of proteinous materials was performed in the 0.3 ml min⁻¹

flow-rate and 2 mL injection size. Then, the column eluent was monitored at 280 nm with the spectrophotometer.

Preparation of Hydrolysate Peptides

Extracted and purified protein was hydrolyzed in SHI conditions. In this regards, the extracted protein with the approximate molecular weight of 22 kDa was digested by Trypsin in enzyme/substrate ratio of 1:50 at 37°C for 18 hours. After that, Chymotrypsin was added and preceded with the same condition. Hydrolysates were centrifuged at 4,000×g for 15 minutes to eliminate both enzymes and non-hydrolyzed proteins, then, passed through 3 and 10 kDa Amicon filters (Billerica, MA, USA) to obtain the smaller than 3 (< 3 kDa), in the range 3 to 10 (3-10), and higher than 10 (> 10 kDa) fractions. Each fraction was collected and examined for its anti-microbial and anti-cancer properties.

Peptide Concentration Determination.

Spectrophotometric assay using O-Phthaldialdehyde (OPA) was used for the determination of the proteolysis degree (Church *et al.* 1983). A fresh OPA solution was prepared daily with 25 mL of 100 mM sodium tetra hydroborate, 2.5 mL of 20% SDS solution (w/w), 1 mL of OPA reagent (40 mg OPA in 1 mL methanol) and 100 µL of β-mercaptoethanol and adjusted to 50 mL with distilled water. 100 µL samples with 1 mL OPA solution were added to the test tube and, after 2 minutes, optical density was read with the spectrophotometer at 340 nm using distilled water as a control.

Anti-Microbial Property Determination

Anti-microbial activity of the protein and the resulting peptide fractions were examined against *E. coli* and *S. aureus*. For this purpose, *E. coli* and *S. aureus* were



cultured in Mueller-Hinton Broth (MHB) at 37°C while the bacterial turbidity adjusted to the 0.5 McFarland (0.08 to 0.13). In order to measure the rate of bacterial growth in presence of the samples, 50 µL of each fraction was added to 200 µL of bacteria culture medium and seeded in 96-well plate. Then, it was kept in the shaker incubator at 37°C for 16 hours.

MIC, which is defined as the lowest antimicrobial concentration with more than 90% decline in bacterial growth, was measured in successive interval times. In this regards, 50 µL of each fraction (< 3 kDa, 3-10, and > 10 kDa) as well as non-hydrolyzed protein were added to separate wells of 96-well plate containing 150 µL of MHB. For each fraction, an aliquot of 100 µL of sample was transferred to the next well and preceded continuously to obtain gradient concentrations. Then, 3-5 µL of MHB containing either *E. coli* or *S. aureus* (0.08 McFarland) was added to each well. Finally, the plate was incubated at 37°C for 18 hours and after that ELISA reader was applied to investigate inhibitory values.

Cell Culture

Human colon adenocarcinoma cell line (SW480) were seeded in appropriate culture medium (RPMI, 10% FBS, 1% Pen/Strep). Cells were plated at a density of 2×10^3 cells/well in a 96-well microtiter plate overnight. Tissue culture plates were incubated at 37°C with humidified atmosphere containing 5% CO₂.

Anti-Tumor Activity Assay

The MTT assay to evaluate the mitochondrial activity was carried out according to the originally described method. The cells were incubated for 24 hours at 37°C and 5% CO₂ and then incubated for different time periods (24, 48, and 72 hours) with each peptide fraction at different concentrations. The MTT dye was

added 4 h prior to completion of the incubation periods. The medium from each well was discarded and the resulting formazan crystals were solubilized by adding 200 µL of dimethylsulphoxide (DMSO) and quantified by measuring absorbance at 570 nm with TECAN Microplate Reader (Infinite® 200 PRO series, Switzerland). Appropriate row or column of wells was left untreated at each time point as the control treatment. The hydrolysate concentration which gives 50% growth inhibition is referred to as the IC₅₀. A simple method for calculation of the IC₅₀ is by linear interpolation between the concentrations above and under 50% inhibition in the dose response curve (= Two flanking points) and is calculated according to the following equation:

$$IC_{50} = \text{EXP}(\text{Ln}(\text{Conc} > 50\%)) \left[\frac{(\text{Signal} > 50\% - 50)}{(\text{Signal} > 50\% - \text{Signal} < 50\%)} \right] \times \text{Ln}(\text{Conc} < 50\% / \text{Conc} > 50\%)$$

Where, conc. is the effective concentration of the desired fraction and signal is related to the inhibition value of the considered sample.

Statistical Analysis

In all experiments, the obtained values were reported as mean ± Standard Deviation (SD) from three independent measurements. One-way Analysis Of Variance (ANOVA) was used to statistically evaluate the obtained values with the level of significance set at probabilities of $P < 0.05$, $P < 0.01$, or $P < 0.001$. SPSS16.0 and Table Curve software were used for the statistical analyses.

RESULTS

Optimum Protein Extraction Method

The high protein content of certain microalgae was one of the reasons to select these organisms as unconventional protein sources. Most of the cultivated microalgae

have a relatively thick cellulosic cell wall which makes the untreated algae practically indigestible to monogastric animals or humans. Among chemical composition and *in vitro* protein digestibility of *S. platensis* biomass, the main feature was their high protein content ($\geq 60\%$). These values are comparable with those obtained in other freshwater microalgal species considered as possible sources of alimentary protein. To improve the whole protein extraction methods by making them faster and more efficient, we studied several different physical and chemical cell disruption and protein extraction methods. According to protein concentration, SDS-PAGE and HPLC results, among the tested methods, the maximum protein content was obtained with liquid nitrogen and lysis buffer extraction method.

From the obtained protein fractions, the fraction with higher concentration in about 20-22 kDa molecular weight was separated for further experiments. Figure 1-A shows an FPLC chromatogram of the extracted whole protein after the sample disruption/cleaning process that shows 3 peaks, and Figure 1-B shows SDS-PAGE gel recorded for these 3 peaks. Although some peaks were detected by FPLC, 3 main

peaks of proteins were obtained from *S. platensis* with 14, 16, and 18 mL retention volumes. From this, the considered extraction method provides a rapid, simple, and efficient approach for isolating the most fraction concentration protein of *S. platensis*.

Enzymatic Hydrolysis of the Extracted Protein

Many anti-microbial and anti-cancer peptides have been discovered from enzymatic hydrolysates of different food proteins, but, so far, there has been no research focused on cheaper algae protein which consists of over 60% protein content. In this section, the extract algae protein hydrolysates were prepared by means of hydrolysis with commercial proteases including Trypsin and Chymotrypsin. The hydrolysis was necessary in order to release potent peptides from the inactive forms of intact algae proteins.

In this regard, the purified protein with about 20-22 kDa molecular weight was hydrolyzed by Trypsin and Chymotrypsin enzymes in SHI conditions to produce bioactive peptides. This hydrolysate had various size ranges of peptides, differentiated as follows: < 3 kDa, 3-10, and

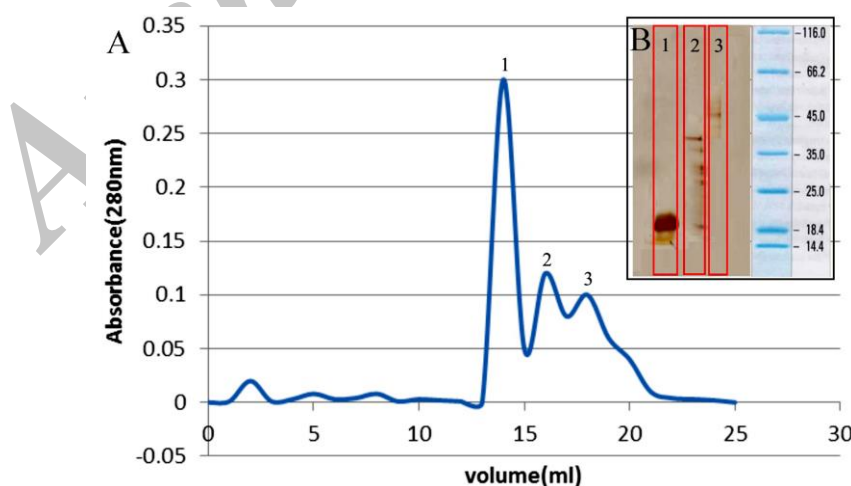


Figure 1 A) FPLC chromatogram for the separation of the proteins by Superdex 75 10/100 GL column using 50 mM sodium acetate and 150 mM sodium chloride buffer at pH 4.8 and B) SDS-PAGE electrophoresis gel for Superdex 75 10/100 GL column chromatography.



> 10 kDa. Since the number of amino acids is necessary for the assessment of peptides functionalities, the fraction with the most significant impact on cell viability was determined.

The degree of hydrolysis measured by the OPA method was raised to $5 \mu\text{g } \mu\text{L}^{-1}$ for all fractions. The optimal concentration of the efficient peptide fractions was determined from the examination of the impact on cells of each fraction at concentrations ranging from 0 to $16 \mu\text{g } \mu\text{L}^{-1}$.

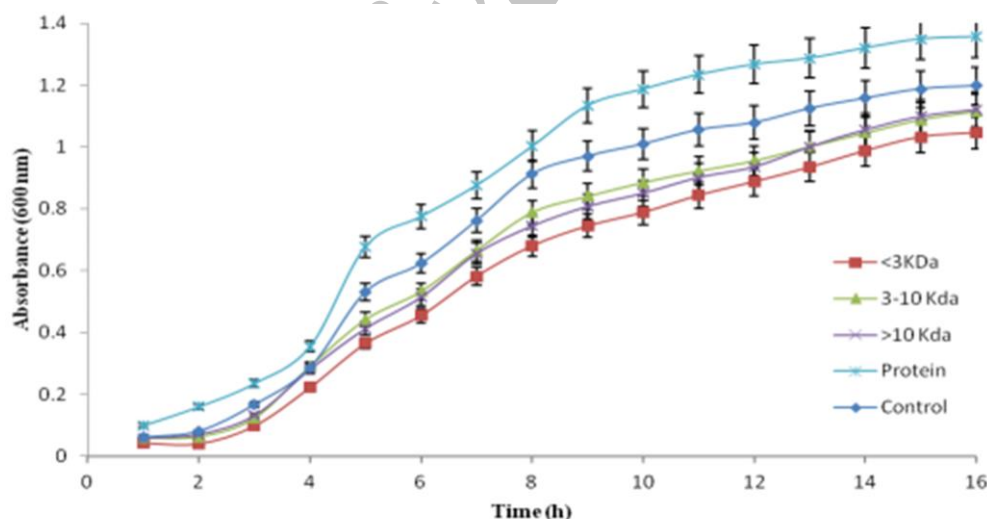
Antibacterial and Aanticancer Activity of Hydrolyzed Peptide Fractions

Anti-bacterial property of peptides was investigated on *E. coli* (Figure 2) and *S. aureus* (Figure 3) compared to 20-22 kDa protein. As shown in Figures 2 and 3, the growth rate of bacteria increased in the presence of non-hydrolyzed protein. This effect in *E. coli* is higher than *S. aureus* that can be demonstrated by the synergistic effect of this protein on the bacterial growth. After that, we used this protein as a source

of bioactive peptide production. Interestingly, the resulting peptides had a negative effect on the growth of these pathogens. The highest inhibitory effect was ascribed to < 3 kDa peptide fraction with 15.2 and 19.6% inhibition after 16 hours for *E. coli* and *S. aureus*, respectively.

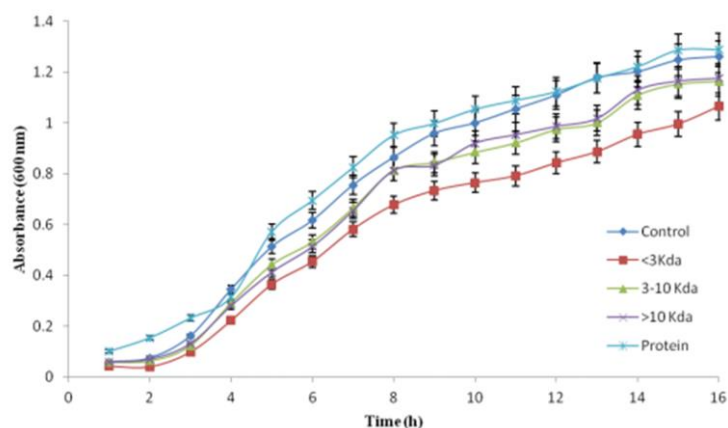
Hence, the < 3 peptide fraction was selected for the subsequent experiments. Since MIC is important in diagnostic laboratories to confirm resistance of microorganisms to an anti-microbial agent and also to monitor the activity of new anti-microbial agents, it was calculated for the most effective peptide fractions. For both bacterial species, it was $625 \mu\text{g mL}^{-1}$ for < 3 kDa peptide fraction.

To investigate the anti-cancer effect of peptide fractions in comparison with purified protein, various concentration of each fraction ($0.01, 0.1, 1, 4, 8, 16 \mu\text{g } \mu\text{L}^{-1}$) was evaluated on the viability of the cultured SW480 cells at 24 and 48 hours time-point. The optimal inhibitory concentration of peptides is shown in Figure 4. As a result, for non-hydrolyzed protein at 48 h time-



Calculated parameters	Control	Non-hydrolyzed protein	< 3 kDa peptide	3-10 kDa peptide	> 10 kDa peptide
Inhibitory effect (%)	0	-15/9	15/2	8/2	7/7
Bacterial doubling time	7/47	7/95	6/59	7/13	6/90

Figure 2. The reduction of growth rate and the doubling time of *E. coli* in presence of different peptide fractions and non-hydrolyzed protein. Statistical analysis showed that, at the 0.05 level, the populations were significantly different.



Calculated parameters	Control	Non-hydrolyzed protein	< 3 kDa peptide	3-10 kDa peptide	> 10 kDa peptide
Inhibitory effect (%)	0	-2/8	19/6	9/6	8/5
Bacterial doubling time	7/14	7/68	6/58	6/88	6/78

Figure 3. The reduction of growth rate and the doubling time of *S. aureus* in presence of different peptide fractions and non-hydrolyzed protein. Statistical analysis showed that, at the 0.05 level, the populations were significantly different.

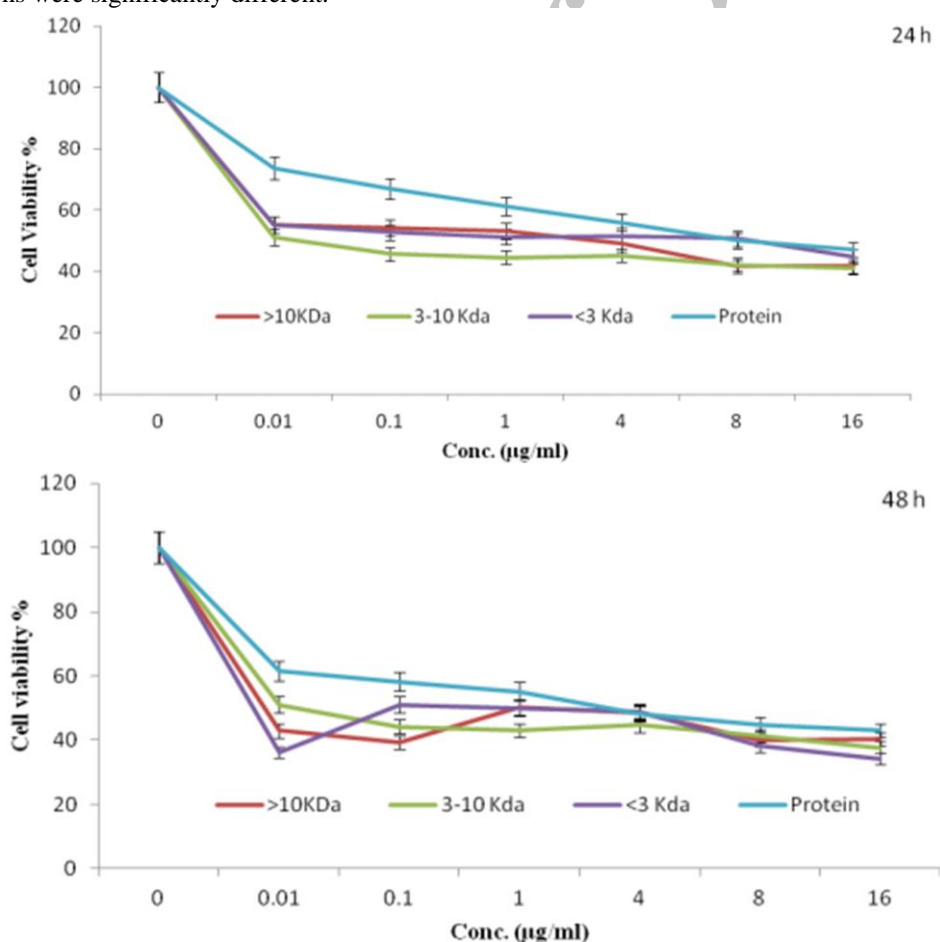


Figure 4. MTT assay for anti-cancer activity of < 3, 3-10, > 10 kDa peptides against non-hydrolyzed protein on SW480 cell line at 24 and 48 h. Statistical analysis showed that, at the 0.05 and 0.01 levels, the populations were significantly different.



point, there was a dose-dependent inhibition of the growth of SW480 cells and the viability of cells in the concentration of $8 \mu\text{g } \mu\text{L}^{-1}$ was 44.9%. In contrast, bioactive peptides showed less than 50% viability even at $0.01 \mu\text{g } \mu\text{L}^{-1}$ that indicated high effectiveness at low concentrations in a time-dependent manner. Among the three peptide fractions, the most effective concentration was the peptides with $< 3 \text{ kDa}$ size in $0.01 \mu\text{g } \mu\text{L}^{-1}$ concentration after 48 hours with the viability of 36%. As a whole, peptide fractions had a better inhibitory effect on the viability of SW480 cells than non-hydrolyzed protein, even at low concentrations.

IC_{50} is an efficient measurement factor of

the inhibitory effect of drugs. As shown in Figure 5, compared with other samples, the IC_{50} for $< 3 \text{ kDa}$ peptides was the lowest value after 48 hours, which resulted in 50% reduction of cell growth at the concentration of $1.04 \mu\text{g } \mu\text{L}^{-1}$.

Introduction of a Prediction Equation for Cell Viability

As a result, $< 3 \text{ kDa}$ peptide fraction was the most effective sample. According to these data and using statistical regression analysis, an equation was obtained to predict cell's behavior in the presence of effective peptide fraction $< 3 \text{ kDa}$. Equation 1

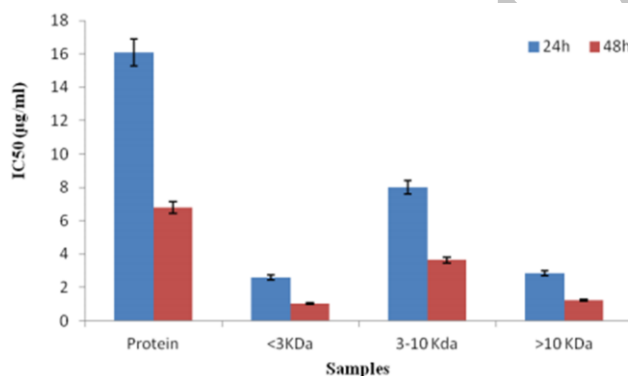


Figure 5. The IC_{50} values for non-hydrolyzed protein and peptide fractions.

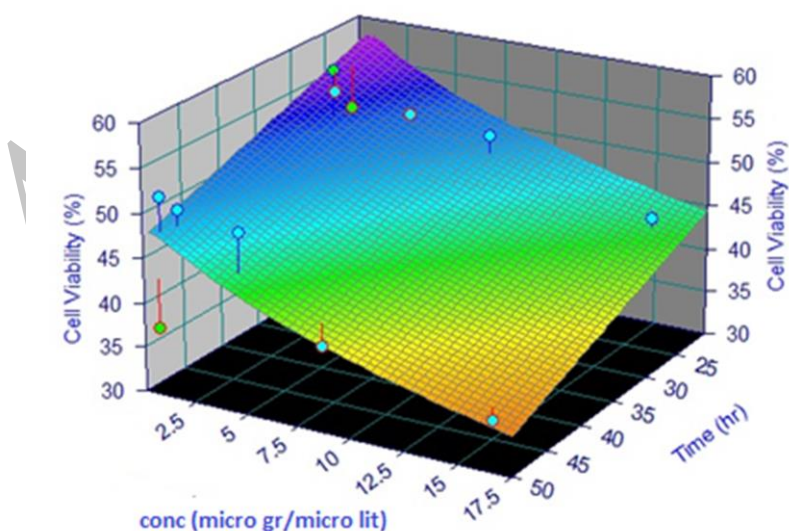


Figure 6. 3D prediction model for cell viability, concentration, and time according to the Equation (1) for $< 3 \text{ KDa}$ sample.

calculates cell viability in different times and concentrations:

$$Z = 64.1 - 0.3X - 1.3\ln Y - 0.9(\ln Y)^2 - 0.07(\ln Y)^3 \quad r^2 = 0.7353 \quad (1)$$

Where, Z is the cell viability percent, X is the time (h) and Y is the peptide concentration ($\mu\text{g } \mu\text{L}^{-1}$). According to this equation, a 3-dimensional prediction model was drawn that could be useful to calculate cell viability of cells. It can accelerate the prediction of results and reduce the number of experiments.

DISCUSSION

The results of this paper showed that enzymatic hydrolysis increased the cytotoxic properties of *S. platensis* abundant protein. In this study, *S. platensis* purified protein was digested with food-grade proteolytic enzymes (Trypsin and Chymotrypsin) to obtain peptide hydrolysates. The initial screening for determination of anti-cancer activity was done employing the MTT assay and the time- and concentration-dependent growth inhibition patterns observed with the < 3 kDa peptide fraction on SW480 cells. This suggests that the fraction could have a better and positive impact on reducing progression of human colon cancer.

Also, the results showed the anti-microbial activity of bioactive peptides specially those < 3 kDa against *E. coli* and *S. aureus*, while purified protein had a synergistic effect on these pathogens. Previous studies also indicated anti-microbial activity of *S. platensis* against *E. coli*; however, only the crude extracts were examined and the results did not introduce any defined and certain anti-microbial substance.

Anticancer peptides is rising interest recently due to their characteristics of multifunction such as high sensitivity, stability, and so on. There have been a few reports on anticancer peptides from food protein, including fish sauce, soy protein, mollusk protein, milk protein, and beef protein (Kim *et al.* 2000; Ben 2005; Leng *et al.* 2005; Wakabayashi *et al.* 2006; Jang *et al.* 2008).

Zhang and Zhang (2013) reported anti-cancer property of *Spirulina* peptides digested by alkalase, pepsin, papain, and trypsin on the whole cell proteins (Zhang & Zhang 2013). They demonstrated that for MCF-7 and HepG2 cells, at $250 \mu\text{g mL}^{-1}$, the maximum inhibitory rate of peptides was 97%. Based on our results, < 3 kDa peptides with IC_{50} of $1.04 \mu\text{g } \mu\text{L}^{-1}$ and after 48 hours were shown to have the highest inhibitory effect on SW480 cell line and could be, therefore, applied to inhibit colon cancer growth. According to previous studies, the peptide with < 6 kDa molecular weight can pass through gastro-intestinal epithelial cells and it is promising that the peptides produced in this research were able to access the damaged cells. Also, Sheih *et al.* (2009) reported that algae waste protein had cytotoxic effect against human gastric cancer cells after hydroxylation by pepsin (Sheih *et al.* 2009)].

Our result suggests that the peptides could be potentially useful adjuncts in the treatment of gastric colon cancer. So, through more complementary tests and evaluating the performance of these peptides after pre-clinical and clinical stages, these peptides can be utilized as a novel drug in the treatment of cancer. Hence, this easily available source makes *Spirulina* protein attractive as a protein source in the future industrial production of functional peptides.

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خواص ضدسرطانی و ضد باکتریایی پپتیدهای حاصل از هیدرولیز پروتئین اسپیرولینا

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چکیده

اسپیرولینا پلاتنسیس ریزجلبکی خوراکی با محتوای پروتئینی بالا (60-70 درصد) می باشد. در حال حاضر، توجه در حای افزایشی به ارزیابی اثر سمیت سلولی پروتئین خوراکی بعد از هیدرولیز به وسیله پروتئاز معدی وجود دارد. متأسفانه، علیرغم تحقیقات گسترده درباره اثر درمانی پروتئین های هیدرولیز شده در فراورده های لبنی، مطالعات بسیار اندکی در حوزه پروتئین ریزجلبک دریایی دردسترس می باشد. بنابراین، این مطالعه تلاش بر بررسی اثرات ضدسرطانی و ضدباکتریایی پروتئینی غالب از اسپیرولینا پلاتنسیس بعد از هیدرولیز توسط آنزیم های تریپسین و کموتریپسین بر روی سلول های سرطان کلون و باکتری های اشرشیا کلی و استافیلوکوکوس اورئوس، به ترتیب، دارد. نتایج نشان داد که پروتئین با وزن مولکولی حدود 20-22 کیلودالتون و پپتیدهای حاصل از آن رشد باکتریایی را کاهش داده و پپتیدهایی با سائز کوچکتر از 3 کیلو دالتون توانایی کاهش قابل توجهی در زنده مانی سلول های SW480 را داشتند. براساس این مطالعه، می توان نتیجه گرفت که اسپیرولینا منبع پروتئینی موثری در تولید صنعتی پپتیدهای عملکردی در آینده خواهد بود.