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Cytotoxicity activity of peptides derived from enzymatic hydrolysis of *Chlorella vulgaris* proteins

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Abstract

Background and Objective: Microalgae are rich sources of bioactive metabolites and one of the major focuses of the pharmaceutical industry is the use of secondary metabolites from plant sources. *Chlorella vulgaris*, a microalga with high economic values, includes a high protein content and significant bioactive compounds and polysaccharides. Therefore, this microalga can be used as a dietary supplement and medicinal product. In this study, inhibition of the growth of colon cancer cells was investigated.

Material and Methods: Proteins of *Chlorella vulgaris* were extracted using enzymatic hydrolysis using proteolytic enzymes of pepsin and Promod (*Bacillus subtilis* protease). Separation of the peptides was carried out using ultrafiltration techniques. Cytotoxic effects of the extracted peptides were assessed using MTT assay on mouse colon tumor cell lines (CT-26).

Results and Conclusion: Results indicated that the pepsin protein hydrolysates (Pep1, Pep2 and Pep3) at a concentration of 1000 mg.ml^{-1} decreased the viability of the CT-26 colon cancer cell line by 24.34%, 36.00% and 40.08%, respectively, while the Promod protein hydrolysates (Pro1, Pro2 and Pro3) decreased the viability by 26.26, 35.91 and 37.13%, respectively. The Pep1 and Pro1 showed the highest cytotoxicity effects ($P < 0.05$). Findings of this study suggest that the bioactive peptides present in *C.* vulgaris may include beneficial functional compounds for cancer prevention.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Microalgae are eukaryotic, microscopic and photosynthetic organisms that serve as rich sources of proteins and nutrients, producing a variety of bioactive compounds. These compounds may possess antimicrobial, anticancer and anti-inflammatory characteristics, contributing to improved health. Demands for microalgae as food supplements are currently increasing [1]. *Chlorella* (*C.*) *vulgaris* is a single-cell green microalga from the family Chlorophyceae that lives in aquatic environments, including freshwater and seawater. This alga includes the highest chlorophyll content within plants and a unique nutritional composition that includes lipids, carbohydrates, fibers, vitamins and proteins. Chlorella is recognized as a superfood that contains 60% of proteins, 18 types of amino acids (AA), 20 vitamins and important minerals such as iron, calcium and potassium [2].

Bioactive peptides, which typically consist of 2-40 AA residues, demonstrate various biological activities depending on the sequence and composition of AAs. To produce protein hydrolysates and extract these peptides from algal proteins, enzymatic hydrolysis is used with proteases such as pepsin and trypsin (4.059, 5.603 mg ml^{-1}, respectively), which significantly affect the bioactivities of the hydrolysates. These hydrolysates are characterized by beneficial attributes such as low molecular weight (LMW), enhanced solubility and decreased odor. Separations such as ultrafiltration and chromatographic techniques are used to achieve purer peptides. The extracted bioactive peptides

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often show a range of therapeutic characteristics, including anticancer, antidiabetic and antioxidant characteristics and include the potential to interact with cellular receptors [3]. Study indicates that short peptides (2-10 AAs) with strong hydrophobic characteristics usually demonstrate high biological activities. These findings highlight considerable potentials of the algae-derived peptides for uses in various industries, including pharmaceutical, cosmetic and food industries [4]. Peptides are short chains of AAs with LMWs that serve defensive and signaling roles in plants. Microalgae can produce active peptides, which can be generated through enzymatic hydrolysis or digestion of microalgal proteins [5].

Cancers are diseases characterized by cells that do not correctly respond to chemical signals sent by other cells. By invading and manipulating normal tissues, these atypical cells include the potential of being lethal. Investigation of chemicals that can induce apoptosis [programmed cell death (PCD)] is important. Cellular desensitization, which occurs when pro-apoptotic signals are diminished or anti-apoptotic signals are enhanced, can lead to a wide range of clinical diseases, including cancers. Therefore, promoting apoptosis is a significant therapeutic target in cancer treatment. Hydrolysates are composed of a complex mixture of free AAs and peptides with molecular weights of 1-7 kDs, as well as lipids and salts at low concentrations, all of which can inhibit cellular proliferation [6]. Chemotherapy, while damaging cancer cells, includes negative effects on normal cells, leading to severe side effects. Therefore, there is a pressing need to discover novel anti-cancer compounds capable of targeting cancer cells without harming healthy cells; thereby, improving patients' quality of life [7].

Dunaliella salina is a halotolerant microalga with high protein contents. Protein hydrolysis was carried out with intestinal proteases such as trypsin and chymotrypsin. Antiproliferative activity of the hydrolyzed peptides was assessed on colon cancer cell lines, respectively. Results demonstrated that peptide fractions smaller than 3 kDa significantly decreased SW480 cell viability [8]. Studies indicate that the development of pharmaceutical molecules from *C. vulgaris* is promising; however, further studies are needed to verify its anti-cancer activities and to convert them into safe and effective drugs for cancer treatment. This study has demonstrated that at a concentration of 500 µg.ml^{-1}, cell mortality significantly increased. Furthermore, bioactive molecules of *C. vulgaris* demonstrated cytotoxic effects against the HepG2 liver-cancer cell line; thereby, affirming its pharmacological activities against the cancer cells [9]. Enzymatic hydrolysis of the cellular proteins of microalgae presents a promising approach to enhance digestibility of proteins, as many microalgae are indigestible by monogastric creatures and humans [10].

Extensive studies have been carried out on the anticancer characteristics of polysaccharides extracted from the green alga of *C .vulgaris*. However, a limited study has been carried out on the anticancer characteristics of bioactive peptides derived from *C. vulgaris.* Commercial proteolytic enzymes were used to hydrolyze *C. vulgaris*. Then, bioactive peptides were isolated using Amicon filters with molecular weight cut-offs of 3 and 10 KDs. Cytotoxicity of the isolated peptides was assessed using MTT assay. Results indicated that proteins derived from *C. vulgaris* possessed significant potentials for uses in pharmaceuticals and functional foods. This shows that proteins derived from algae demonstrate substantial potentials for use in pharmaceuticals, cosmetics and functional foods. Therefore, the present review focused on bioactive peptides extracted from *Chlorella* with an emphasis on cytotoxic effects on mouse colon cancer cell lines.

2. Materials and Methods

2.1. Methods

The *C. vulgaris* strain was achieved from the Caspian Sea Ecology Research Institute, Sari, Iran. Enzymatic hydrolysis was carried out using two types of commercial protease enzymes of pepsin (EC 3.4.23.1) and Promod 971MDP. The CT-26 colon-cancer cell line was achieved from the Pasteur Institute of Iran, North Research Center.

2.2. Preparation and production of *C. vulgaris* powder

Cultivation of this microalga was carried out using Bold basal (BB) media [11] to achieve the necessary biomass volume. Then, general culture media (TMRL) supplemented with sodium bicarbonate were used in 254-ml containers. The initial biomass weight of the algae was maintained at 0.1 g.l^{-1} in all containers. The initial cultivation conditions included pH of 6.8, temperature of 25 °C, white light with an intensity of 1444 lux and light schedule of 16-h dark and 8-h light. Water temperature was set at an average of 20 °C, salinity at 25 ppt and pH at 7.5-8. Algae in the culture tanks were constantly aerated using aeration pumps. Furthermore, the containers were continuously aerated. For drying of *C. vulgaris*, an oven-drying method was used. Initially, the cultured algae were centrifuged and the concentrated pellet was spread in a thin layer on glass plates. Using electric oven, samples were dried at 60 °C for 12 h to reach a constant weight [12].

2.3. Analysis of the composition of *C. vulgaris* powder

Chemical composition of the samples, including moisture, crude protein, fat and ash, was assessed based on the standard AOAC method [13] with minor modifications. To assess the moisture content, samples were transferred into an oven with a temperature of 105 °C to reach a constant weight. To assess the ash, dry sample was transferred into a porcelain crucible and burned at 550°C for 5 h using furnace

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(Model FX 118-30, Nabertherm, Germany). Protein content was assessed using Kjeldahl method with a nitrogen conversion factor of 6.25 (Model S3, Behr, Germany). Additionally, total fat was assessed using Soxhlet apparatus with hexane as solvent (Model R 254 S, Behr, Germany) [14]. Carbohydrate content in the dry biomass was assessed using colorimetric method [15].

2.4. *Chlorella vulgaris* enzymatic hydrolysis

Enzymatic hydrolysis of *C. vulgaris* was carried out using a method described by Liu et al. [15] with minor modifications. First, dried *C. vulgaris* was mixed with distilled water (DW) at a ratio of 1:20 (VW). This mixture was frozen at low temperatures (-20 °C) for 24 h, then it was gradually thawed for 24 h using refrigerator and then refrozen. Enzymatic hydrolysis was carried out using two types of commercial protease enzymes of pepsin (EC 3.4.23.1) and Promod 971MDP. This enzyme acted at 35 °C and pH 3 for 2 h. The Promod enzyme, derived from a microbial source (*Bacillus subtilis*), was used at 40 °C and pH 7 for 1 h. In the two examples, the ratio of enzyme to substrate was set at 1:100. Following hydrolysis, mixtures were heated to 85 °C for 15 min to deactivate the enzymes. Then, samples were isolated by spinning in a centrifuge at 8000 g for 15 min. Hydrolyzed products were prepared through lyophilization and were stored at -20 °C until use [16].

2.5. Degrees of hydrolysis

The O-phthaldialdehyde (OPA) was used to assess concentrations of peptides following a protocol developed by Moaveni et al. [17]. The OPA reagent can react with primary amines (peptide functional groups) in presence of β-mercaptoethanol. Then, 50 µl of the sample were mixed with 500 µl of the OPA reagent using 96-well plate and incubated for 2 min at room temperature (RT). Then, absorbance was measured at 340 nm using ELISA plate reader (Hiperion, Germany). The Eq. 1 shows peptone standard curve of 0-30 mg.ml^{-1} [17].

$$DH\ (\%) = \frac{\text{Soluble Protein of Hydrolysate}}{\text{Total Protein of Sample}} \quad \text{Eq. 1}$$

2.6. Assessing protein yield

Protein concentration was assessed using Biuret method. Briefly, 0.1 ml of the protein solution was mixed with 0.4 ml of Biuret reagent and set for 30 min at RT. Optical density was assessed at 540 nm using UV-visible spectrophotometer (model U-2001, Hitachi, Tokyo, Japan). Bovine serum albumin (BSA) was used as the protein standard. Protein concentration was assessed using calibration curve of Eq. 2:

$$y = 19.256x - 0.106\ (R^2 = 0.999) \quad \text{Eq. 2}$$

Where, y indicated the protein concentration (mg.ml^{-1}) and x represented the optical density (OD) at 540 nm. Protein yield was calculated using Eq. 3:

$$Y = C \times V/M \quad \text{Eq. 3}$$

Where, Y was the protein yield (mg.g^{-1}), C was the protein concentration (mg.ml^{-1}), V was the volume of the protein extract (8 ml) and M was weight of the residual microalgae (g) [18].

2.7. Assessment of the hydrolyzed protein yield

Yield of the hydrolyzed *C. vulgaris* protein was assessed based on a method described by Islam et al. [14] and this yield was calculated using Eq. 4:

$$\text{Yield}\ (\%) = \frac{\text{Weight of protein hydrolysate powder (g)}}{\text{Weight of raw material (g)}} \times 100 \quad \text{Eq. 4}$$

2.8. Ultrafiltration

Two filters (Millipore, USA) with molecular weights of 3 and 10 kDs were used. Ultrafiltration process was divided into three fractions of Pep1 and Pro1 < 3 kDa, Pep2 and Pro2 3-10 kDa, and Pep3 and Pro3 >10 kDa. Antioxidant and anticancer characteristics of all fractions were investigated [16].

2.9. Cell culture

The cell line was cultured in RPMI 1640 media supplemented with 10% of heat-inactivated FBS and 1% of penicillin. Temperature, CO_2 concentration and humidity in the culture were set at 37 °C, 5% and 95%, respectively. When cells in the culture flasks reached a density of 80-90%, 0.05% trypsin solution was introduced to the cells. Then, cells were centrifuged at 130 g for 5 min using centrifuge. Following cell count using cytology glass slides, cells were stored in a refrigerated microplate to create a mouse model for studying effects of the supernatant [19].

2.10. The cytotoxicity assay

The mortality rate of cancer cells was assessed using MTT assay. Briefly, 100 µl of each cell group, which was adjusted with an initial culture of 1.2×10^4 cells per ml, were transferred into the wells of 96-well plates. Then, RPMI culture media containing 10% FBS were used as the control. The *C. vulgaris* polysaccharides in the concentration range of 62.5-1000 mg.ml^{-1} were added to the wells and cisplatin (Cis-Plt) was used as a positive control. Cells were incubated at 37 °C for 48 h. After incubation, culture media of each well were replaced with 50 µl of MTT reagent (Promega, USA) in each well. Wells were set to incubate at 37 °C for another 4 h in a humidified environment with 5% CO_2. The control cells received only culture media without any suspension. Then, plate was centrifuged at 800 g for 5 min to eliminate MTT that was not converted and the supernatant was suctioned out. Formazan crystals in every well were dissolved using 150 µl of DMSO. Quantity of

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purple formazan was assessed by measuring the absorbance at 540 nm. Treated cells' viability was quantified as a percentage relative to the control cells. Viability rate and inhibition ratio were assessed using Eq. 5.

$$\text{Viability rate (\%)} = \frac{\text{Mean optical density of control cells}}{\text{Optical density of treated cells}} \times 100$$

Eq. 5

(Mean optical density of the control cells / optical density of the treated cells) * 100

All assessments were carried out in triplicate [19].

2.11. Assessment of IC50

Calculation of the IC50 for each analytical sample was carried out using the following method. At six various points, concentration of the protein samples (x) was plotted against inhibition ratios (y) and a regression line (y = ax + b) was created. It is noteworthy that the regression line was not necessary to go through the origin. More precisely, since the inhibition curve was not completely linear and included a slight curve, the IC50 value was calculated by connecting two points close to the concentration at 50% inhibition with a straight line through an interpolation method. Two points representing 50% inhibition were chosen and a regression line (y = ax + b) was plotted using these points for analysis. To calculate the x value (sample concentration), the y value was set to 50 in the regression equation of y = ax + b [20].

2.12. Morphological assessment of the cancer cell lines

To assess cellular morphology changes, May-Grunwald-Giemsa staining method was used. The CT-26 cells were transferred into Lab-Tek chambers at a concentration of 5×10^4 cells/ml (450 µl per well). After 24 h, culture media were out from the chambers and cells were subjected to various peptides at concentrations of 62.5 and 1000 $mg.ml^{-1}$. These peptides were used in combination at a 1:1 ratio or administered separately. For the positive control, 10 µM of 5-fluorouracil (FU-5) and 10 µM of cisplatin (Cis-Plt) were used. The peptide fractions of anti-cancer solutions were prepared in media with 10% of FBS. After 48 h, cells were dyed with May-Grunwald-Giemsa stain. Cells with stains were then assessed and studied using Olympus BX51 System Microscope (Olympus, Japan) [21].

2.13. Statistical analysis

Experiments were carried out three times and results were recorded. For data analysis, one-way ANOVA was used and Duncan's multiple range tests were used at a 5% significance level for the comparison of means. The mean values and standard deviations were calculated using Excel 2010 software. Additionally, data were analyzed using SPSS v.18 and the graphs were generated using Excel.

3. Results and Discussion

3.1. Proximate composition of the biochemical compounds

The initial protein content of *C. vulgaris* was 47.02% ±0.05. Protein constitutes a significant portion of algae, typically accounting for 50-70% of their dry weight (DW). Table 1 shows the approximate assessment of the biochemical compounds in *C. vulgaris* algae. Derbel et al. [22] assessed composition of *Rhodomonas* biomass collected at the early stationary phase. The achieved results showed that *Rhodomonas* sp. accumulated 34.5 $mg.100^{-1}$ mg protein, 14.18 $mg.100^{-1}$ mg carbohydrate, 13 $mg.100^{-1}$ mg lipid, 15.73 $mg.100^{-1}$ mg ash and 0.46 $mg.100^{-1}$ mg chlorophyll [22].

Table 1. Biochemical compounds of *Chlorella vulgaris*

Biochemical Composition	*C. vulgaris* (%)
Proteins	0.05±47.02
Carbohydrates	0.09±19.08
Lipids	0.07±12.52
Humidity	0.03±4.67
Ash	0.04±7.45

3.2. *Chlorella vulgaris* hydrolyzed protein cytotoxicity

Cytotoxic effects of the hydrolyzed proteins were assessed and the percentage of cell viability for cells treated with hydrolyzed proteins from pepsin and Promod (with molecular weights < 3 kDa) at various concentrations (62.5, 125, 250, 500 and 1000 µg/ml) is present in Figure 1. Results achieved from the MTT assay indicated increases in the concentration of hydrolyzed pepsin and Promod proteins, leading to decreases in the viability of cancer cells. Additionally, hydrolyzed proteins from *C. vulgaris* resulted in decreased cellular activities. Specifically, the IC50 values for hydrolyzed pepsin and promod (Pep1 and Pro1) proteins for this cell line were recorded as 324.78 and 459.81 µg/ml after 48 h, respectively ($P < 0.05$) (Figure 1a). The Pep1 and Pro1 less than 3 KDa included further biological activities. As a positive control for the experiment, concentrations of 0.031 to 0.5 µg/ml of cisplatin (Cis-Plt) were used. The major mechanism of action of anti-cancer peptides is to cause cell membrane fragmentation or apoptosis by depolarization of the cell membrane, leading to failure of tumor cells to maintain normal osmotic pressure [23].

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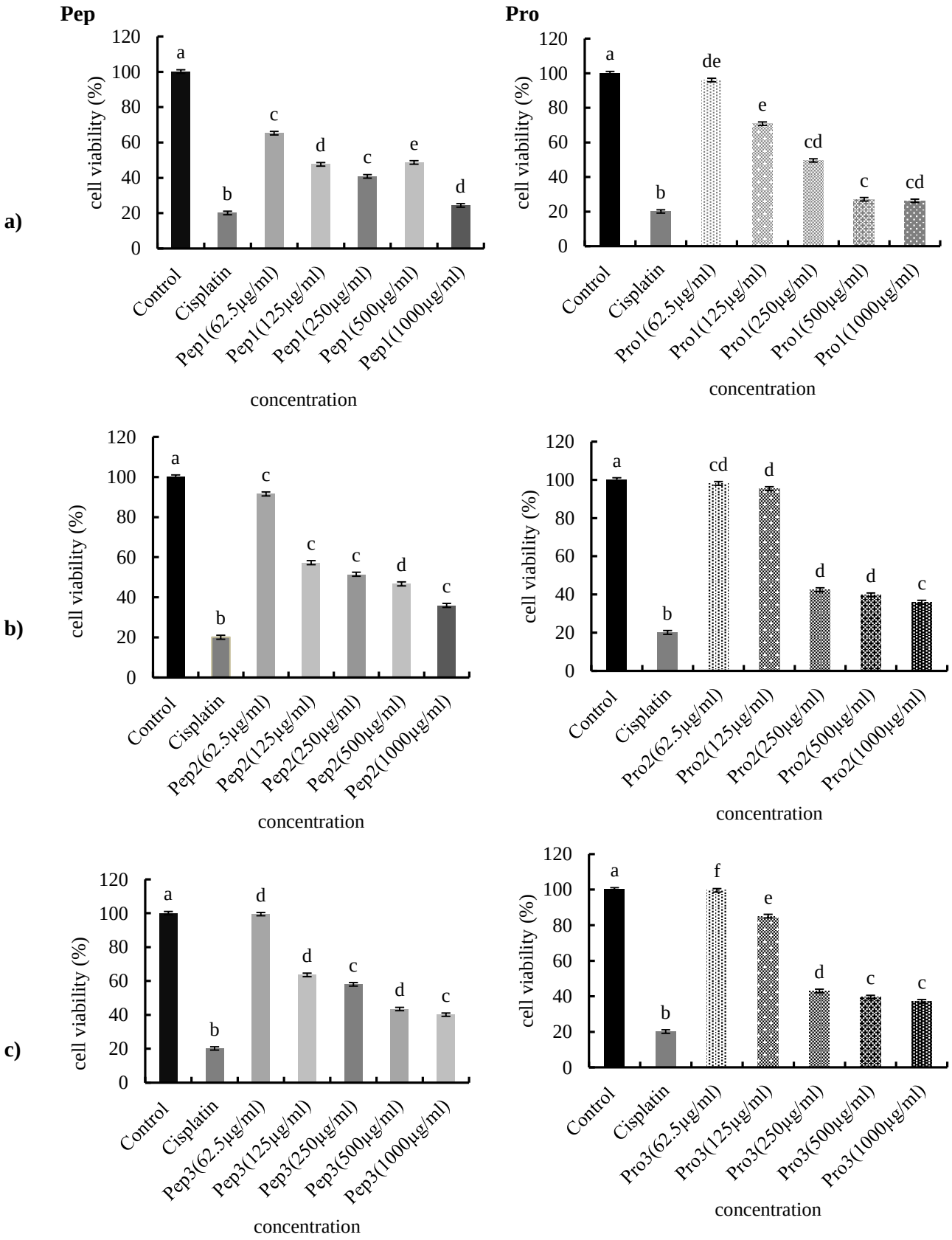

Figure 1. The MTT assay to assess cytotoxicity activity of *Chlorella vulgaris* hydrolyzed protein with a) Pep1 and Pro1 < 3 kDa, b) Pep2 and Pro2 of 3-10 kDa and c) Pep3 and Pro3 > 10 kDa on CT-26 cells. Different letters indicate significant differences between each group.

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Viability percentage of cells treated with hydrolyzed proteins from pepsin and Promod (with molecular weights of 10-3 kDs) at various concentrations (62.5, 125, 250, 500 and 1000 µg/ml) is shown in Figure 1. Results from the MTT assay indicated that hydrolyzed proteins from *C. vulgaris* decreased cellular activity with IC50 values for the hydrolyzed proteins of pepsin and promod (Pep2 and Pro2) for this cell line recorded as 543.40 and 650.33 µg/ml after 48 h, respectively ($P < 0.05$) (Figure 1b). The survival percentage of cells treated with hydrolyzed proteins from pepsin and promod (> 10 kDs) at various concentrations (62.5, 125, 250, 500 and 1000 µg/ml) is illustrated in Figure 1. The MTT assay results indicated that hydrolyzed proteins from *C. vulgaris* contributed to decreases in cellular activity. Additionally, the IC50 values of the hydrolyzed proteins from pepsin and promod (Pep3 and Pro3) for this cell line were recorded as 679.15 and 802.72 µg/ml after 48 h, respectively ($P < 0.05$) (Figure 1c).

The MTT assay indicated that the peptide fraction with a molecular weight of less than 3 kDa demonstrated a positive effect and showed a further effective inhibitory action on cancer cells, compared to other protein fractions. Protein composition and molecular weight play important roles in the digestibility of microalgae proteins [24]. Smaller peptides are usually more easily absorbed through the cell membrane. This characteristic allows them to quickly reach tissues and cells and initiate their biological effects. They are further resistant to enzymatic degradation, which can help increase their half-life in the body [25].

Pepsin hydrolysates of Pep1, Pep2 and Pep3, at a concentration of 1000 µg, decreased survival rates of the CT-26 colon-cancer cell line by 24.34, 36.00 and 40.08%, respectively. Moreover, the Promod hydrolysates of Pro1, Pro2 and Pro3 decreased survival rates of the CT-26 cell line by 26.26, 35.91 and 37.13%, respectively, indicating their cytotoxic effects. A study by Sarvaysh and Rajasulochana [9] demonstrated that time and concentration-dependent growth inhibitory patterns with peptide fractions < 5 KDa yielded better results in HCT-116 cells, compared to HTB-26, as shown via the MTT method. In studies carried out by Kunte et al. [26], it was observed that protein extracts from *C. minutissima* suppressed the overexpression of metalloproteinases of MMP-2 and MMP-9 in HepG2 cells. It is suggested that *Chlorella* includes significant potentials in cancer treatment.

Creating a therapeutic molecule from natural sources is a difficult process that needs rigorous scientific validation. This process involves locating the active ingredients, assessing their pharmacological characteristics and carrying out clinical studies to investigate safety and efficacy. It is important to highlight that creating a therapeutic molecule is a costly and time-consuming operation [9]. In a study on identifying the substance that was responsible for the anticancer effects of *C. vulgaris* against adenocarcinoma stomach cancer cells, AGS demonstrated that *C. vulgaris* peptide fraction with LMW included strong dose-dependent cytotoxic activities and stimulated cell cycle arrest at post-G1 in AGS cells [27]. The peptide fraction possessed cell-specific effects as no significant inhibitive effect was observed against human colon adenocarcinoma cells of C2BBel, human hepatoblastoma cell lines of Hep G2 and human cervical epithelioid carcinoma cells of Hela. Within the peptides in the fraction, a short peptide with an AA sequence of VECYGPNRPQF showed effective antiproliferative, antioxidant and anti-inflammatory activities [28].

The correlation between cytotoxicity of Cis-Plt and *C. vulgaris* bioactive peptides is shown in Fig. 2. The correlation between bioactive peptides from *C. vulgaris* and cell viability was investigated, yielding significant results for pepsin (Pep1) protein hydrolysate ($R2 = 0.6965$), Promod (Pro1) protein hydrolysate ($R2 = 0.5850$) and Cis-plt ($R2 = 0.8796$). As illustrated in Fig. 2, bioactive peptides with a molecular weight < 3 kDa affected the antioxidant capacity of *C. vulgaris*. Pepsin and Promod are protease enzymes, but pepsin is further concerned with the initial digestion of proteins in an acidic environment. Promods may include various characteristics and functions and may be involved in various stages of protein digestion. Slope of the line between the pepsin (Pep1), Promod (Pro1) protein hydrolysate and Cis-plt included -0.0325, -0.0515 and -6.3617 for protein hydrolysates and Cis-plt, respectively. A negative slope of the line means that as the drug inhibition increases, the percentage of cancer cell viability decreases. This indicates that the drug is effective in inhibiting the growth of cancer cells.

3.3. Cell morphology of the pepsin and Promod hydrolysates

Cellular morphology of the pepsin and Promod hydrolysates from *C. vulgaris* with three molecular weight ranges of less than 3 kDs, 3-10 kDs and less than 10 kDs at various concentrations (62.5 and 1000 $\mu g.ml^{-1}$) is present in Fig. 3A, B.

Morphological changes in mouse colon cancer cells induced by hydrolyzed proteins from *C. vulgaris* are present in Fig. 3. Pepsin and Promod hydrolysates inhibited the proliferation of CT-26 cells. After 48 h of incubation, apoptotic cells began to detach from the monolayer and formed spherical shapes of various sizes. The strongest anti-proliferative effect was observed on CT-26 mouse colorectal cancer cells treated with *Chlorella* protein hydrolysate fractions at concentrations of 500 and 1000 $\mu g.ml^{-1}$ and with a molecular weight of less than 3 kDa (Fig. 3). Simultaneously, various concentrations of cisplatin (0.031-0.5) decreased the number of mouse colon cancer

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cells and included a significant effect on their morphology. In a study by Lemizek and Rzeski [21], it was shown that barley and *Chlorella* extracts, alone or in combination at the two concentrations of 500 and 1000 µg.ml^{-1}, did not affect the morphology or growth pattern of human CCD841 epithelial cells. A similar effect of *Chlorella* and mixture of young green barley on colon epithelial cells have been reported for the first time in this study.

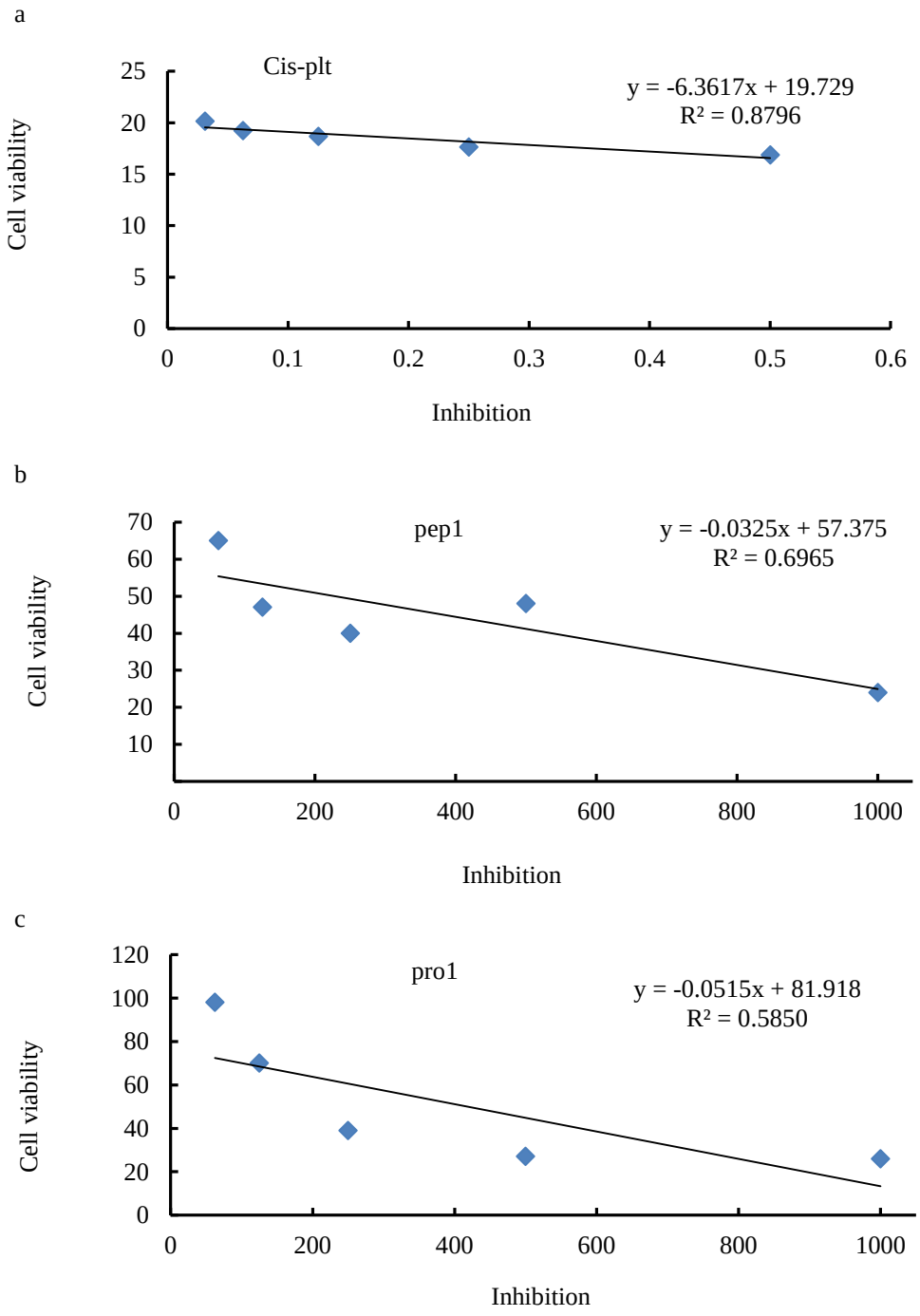

Figure 2. Correlation diagrams between the cytotoxicity of Cis-Plt and *Chlorella vulgaris* bioactive peptides using regression analysis. a) Cis-Plt, b) Pep1 (pepsin protein hydrolysate) and c) Pro1 (Promod protein hydrolysate)

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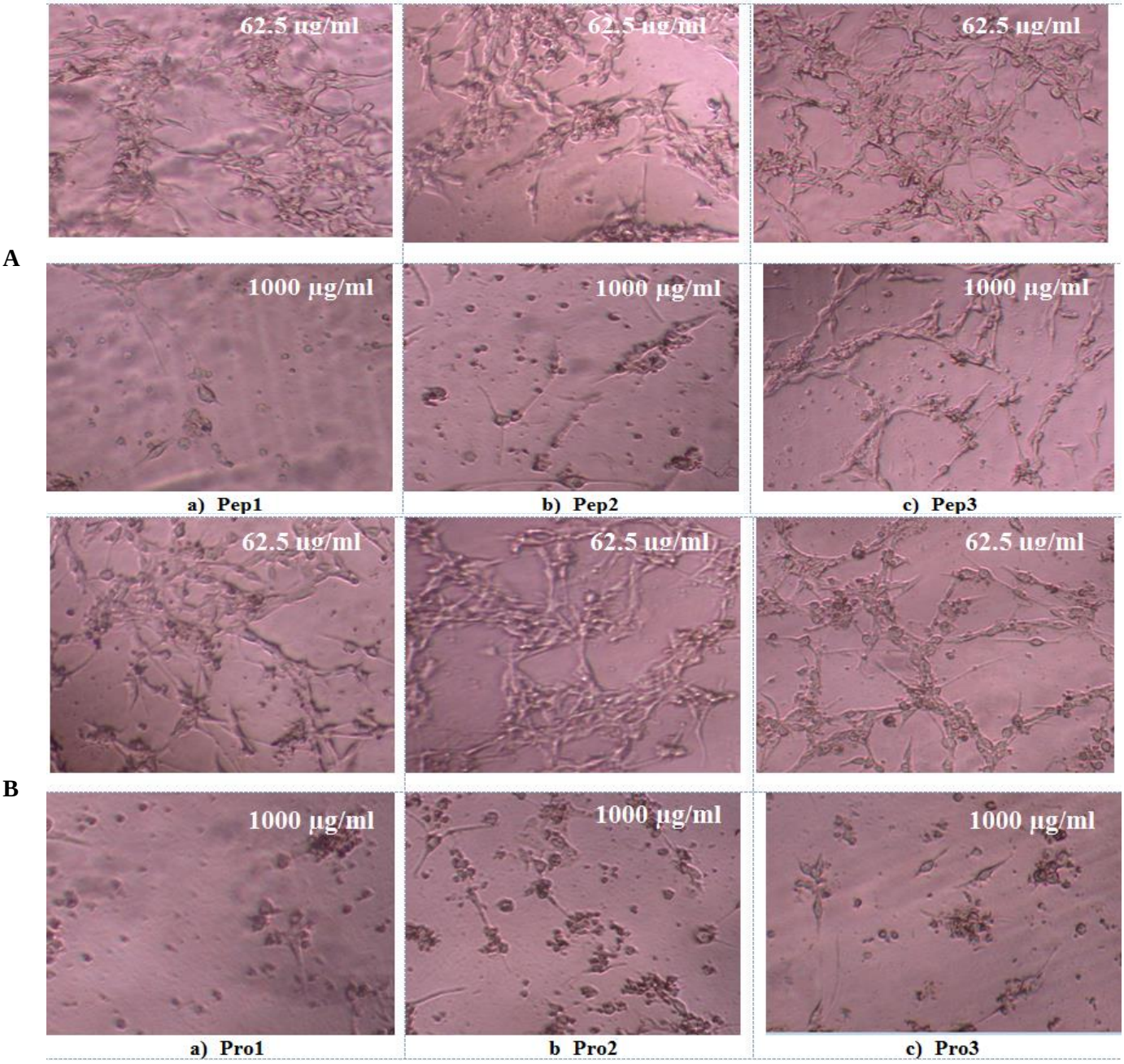

Figure 3. Phase-contrast micrographs of *Chlorella vulgaris*-derived cytotoxicity of bioactive peptides. (a) Cells treated with Pep1 and Pro1, (b) cells treated with Pep2 and Pro2 and (c) cells treated with Pep3 and Pro3 at two concentrations (62.5 and 1000 µg.ml^{-1}).

4. Conclusion

Extraction of bioactive peptides from *C. vulgaris* through proteolytic enzymatic hydrolysis using pepsin and Promod has demonstrated significant anti-cancer characteristics, specifically in decreasing survival rate of the CT-26 colon cancer cell line. Data indicate that pepsin and Promod-derived protein hydrolysates possess cytotoxic effects and show the most decreases in cell viability. These results highlight the potential of *C. vulgaris*-derived peptides and suggests that the effective compounds of *Chlorella* can be used as a biological anticancer drug in treatment of colon cancer after further *in vivo* assessments in mice. The major advantages of biological drugs include enhancing the immune system, lack of side effects and high efficacies. *Chlorella* derived peptides as functional compounds are suggestions for their practical uses in food science such as their uses as natural additives in food products, production of food supplements, incorporation into dairy products such as yogurts and cheeses and uses in livestock and poultry feeds. Further studies are warranted to elucidate underlying mechanisms of action and assess the therapeutic potential of these bioactive peptides in clinical settings.

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5. Acknowledgements

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6. Conflict of Interest

The authors declare no conflict of interest.

References

1. Skjanes K, Aesoy R, Herfindal L, Skomedal H. Bioactive peptides from microalgae: Focus on anticancer and immunomodulating activity. Physiol Plant. 2021; 173(2): 612-623. https://doi.org/10.1111/ppl.13472
2. Jayshree A, Jayashree S, Thangaraju N. *Chlorella vulgaris* and *Chlamydomonas* reinhardtii: Effective antioxidant, antibacterial and anticancer mediators. Indian J Pharm Sci. 2016; 78(5): 575-581. https://doi.org/10.4172/pharmaceutical-sciences.1000155
3. Liu J, Bai X, Fu P. In silico and in vitro assessment of bioactive peptides from *Arthrospira platensis* phycobiliproteins for DPP-IV inhibitory activity, ACE inhibitory activity, and antioxidant activity. J Appl Phycol. 2022; 34(3): 1497-1511. https://doi.org/10.1007/s10811-022-02732-z
4. Leong YK, Chang JS. Proteins and bioactive peptides from algae: Insights into antioxidant, anti-hypertensive, anti-diabetic and anti-cancer activities. Trends Food Sci Technol. 2024; 104352. https://doi.org/10.1016/j.tifs.2024.104352
5. Santa M. Brazil RS. Handbook of Microalgae-Based Processes and Products, In: Acquah C, Tibbetts SM, Pan S, Udenigwe C. Nutritional Quality and Bioactive Properties of Proteins and Peptides from Microalgae. Academic Press. 2020: pp. 493-531. https://doi.org/10.1016/B978-0-12-818536-0.00019-1
6. Brodin P. Davis M.M. Human Immune System, In: Ghosh S, Bhattacherjee R, Banerjee D. Cell Death and Nanoparticles. 2022: pp. 71. https://doi.org/10.1515/9783110655858-005
7. Yao W, Qiu HM, Cheong KL, Zhong S. Advances in anti-cancer effects and underlying mechanisms of marine algae polysaccharides. Int J Biol Macromol. 2022; 221: 472-485. https://doi.org/10.1016/j.ijbiomac.2022.09.055
8. Darvish M, Jalili H, Ranaei-Siadat SO, Sedighi M. Potential cytotoxic effects of peptide fractions from *Dunaliella salina* protein hydrolyzed by gastric proteases. J Aquat Food Prod. 2018; 27(2): 165-175. https://doi.org/10.1080/10498850.2017.1414095
9. Sarvaysh Y, Rajasulochana P. A novel potent drug molecule from *Chlorella vulgaris* for the control of cancer cells. Eur Chem Bull. 2023; 12(4): 3098-3109.
10. Sedighi M, Jalili H, Ranaei, SSO, Amrane A. Potential health effects of enzymatic protein hydrolysates from *Chlorella vulgaris*. Appl Food Biotechnol. 2016; 3 (3):160-169. https://doi.org/10.22037/afb.v3i3.11306
11. Munir N, Imtiaz A, Sharif N, Naz S. Optimization of growth conditions of different algal strains and determination of their lipid contents. J Anim Plant Sci. 2015; 25(2): 546-553.
12. Razi N, Shamsaie Mehrgan M, Hosseini Shekarabi S.p. A comparative study of different drying methods on some proximate composition and pigments of marine microalgae (Isochrysis galbana). Aqua Sci. 2019; 7(2): 12-20.
13. Association of Official Analytical Chemists – AOAC. Official Methods of Analysis of AOAC International (18th ed.). Washington: AOAC. 2005.
14. Islam MS, Hongxin W, Admassu H, Noman A, Ma C, Wei F. Degree of hydrolysis, functional and antioxidant properties of protein hydrolysates from Grass Turtle (Chinemys reevesii) as influenced by enzymatic hydrolysis conditions. Food Sci Nutr. 2021; 9: 4031-4047. https://doi.org/10.1002/fsn3.1903
15. Hussain J, Wang X, Sousa L, Ali R, Rittmann BE, Liao W. Using non-metric multi-dimensional scaling analysis and multi-objective optimization to evaluate green algae for production of proteins, carbohydrates, lipids, and simultaneously fix carbon dioxide. Biomass Bioenergy. 2020; 141:p.105711. https://doi.org/10.1016/j.biombioe.2020.105711
16. Liu Y, Qi Y, Wang Q, Yin F, Zhan H, Wang H, Liu B, Nakamura Y, Wang J. Antioxidative effect of *Chlorella pyrenoidosa* protein hydrolysates and their application in Krill oil-in-water emulsions. Mar Drugs. 2022; 20(6): 345. https://doi.org/10.3390/md20060345
17. Moaveni S, Salami M, Khodadadi M, McDougall M, Emam-Djomeh Z. Investigation of S. limacinum microalgae digestibility and production of antioxidant bioactive peptides. Lwt. 2022; 154: 112468. https://doi.org/10.1016/j.lwt.2021.112468
18. Lu K, Zhao X, Ho SH, Ma R, Xie Y, Chen J. Biorefining and the functional properties of proteins from lipid and pigment extract residue of *Chlorella pyrenoidosa*. Mar drugs. 2019; 17(8): 454. https://doi.org/10.3390/md17080454
19. Shafay SE, El-Sheekh M, Bases E, El-Shenody R. Antioxidant, antidiabetic, anti-inflammatory and anticancer potential of some seaweed extracts. Food Sci Technol. 2021; 42: 20521. https://doi.org/10.1590/fst.20521
20. Xiao F, Xu T, Lu B, Liu R. Guidelines for antioxidant assays for food components. Food Front. 2020; 1(1): 60-69. https://doi.org/10.1002/fft2.10
21. Lemieszek MK, Rzeski W. Enhancement of chemopreventive properties of young green barley and Chlorella extracts used together against colon cancer cells. Ann Agric Environ Med. 2020; 27(4): 591. https://doi: 10.26444/aaem/130555
22. Derbel H, Elleuch J, Mahfoudh W, Michaud P, Fendri I, Abdelkafi S. In Vitro Antioxidant and Anti-Inflammatory Activities of Bioactive Proteins and Peptides from Rhodomonas sp. Appl Sci. 2023; 13(5): 3202. https://doi.org/10.3390/app13053202
23. Xie M, Liu D, Yang Y. Anti-cancer peptides: Classification, mechanism of action, reconstruction and modification. Open biology. 2020; 10(7): 200004.
24. Sedighi M, Jalili H, Darvish M, Sadeghi S, Ranaei-Siadat SO. Enzymatic hydrolysis of microalgae proteins using serine proteases: A study to characterize kinetic parameters. Food Chem. 2019; 284: 334-339. https://doi.org/10.1016/j.foodchem.2019.01.111
25. Oroojalian F, Beygi M, Baradaran B, Mokhtarzadeh A, Shahbazi MA. Immune cell Membrane Coated biomimetic nanoparticles for targeted cancer therapy. Small. 2021; 17(12): 2006484. https://doi.org/10.1002/smll.202006484
26. Kunte M, Desai K. The protein extract of *Chlorella minutissima* inhibits the expression of MMP-1, MMP-2 and MMP-9 in cancer cells through upregulation of TIMP-3 and down regulation of c-Jun. Cell J. 2018; 20(2): 211-19. https://doi.org/10.22074/cellj.2018.5277
27. Sheih IC, Fang TJ, Wu TK, Lin PH. Anticancer and antioxidant activities of the peptide fraction from algae protein waste. J Agric Food Chem. 2010; 58(2):1202-7. https://doi:10.1021/jf903089m
28. Adzahar NS, Basri DF, Latif ES, Sallehudin NJ. In vitro and in vivo cytotoxic effects of Chlorella against various types of cancer. IIUM Medical Journal Malaysia. 2021; 20(1). https://doi.org/10.31436/imjm.v20i1.1765

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فعالیت سمیت سلولی پپتیدهای حاصل از آبکافت آنزیمی پروتئین‌های *کلرلا ولگاریس*

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- کلرلا ولگاریس
- ریزجلبک

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چکیده

سابقه و هدف: ریزجلبک‌ها منابع غنی متابولیت‌های زیست‌فعال می‌باشند و یکی از تمرکزهای اصلی صنعت داروسازی استفاده از متابولیت‌های ثانویه حاصل از منابع گیاهی است. *کلرلا ولگاریس*، ریزجلبکی با ارزش اقتصادی بالا، حاوی میزان بالایی پروتئین و ترکیبات زیست‌فعال و پلی ساکارید است. بنابراین می‌توان از این ریزجلبک به‌عنوان مکمل غذایی و فرآورده دارویی استفاده کرد. در این مطالعه مهار رشد سلول‌های سرطانی روده بزرگ مورد بررسی قرار گرفت.

مواد و روش ها: پروتئین *کلرلا ولگاریس* به۸روش آبکافت آنزیمی و با استفاده از آنزیم‌های پروتئولیتیک پپسین و پرومود (پروتئاز *باسیلوس سوبتیلیس*) استخراج شد. جداسازی پپتیدها با استفاده از تکنیک‌های فراپالایی[1] انجام شد. اثرات سمیت سلولی پپتیدهای استخراج شده با استفاده از روش MTT بر روی رده‌های سلولی سرطان کولون موش (CT-26) ارزیابی شد.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد که ترکیبات حاصل از آبکافت پروتئین پپسین (Pep1، Pep2 و Pep3) در غلظت $mg.ml^{-1}$ ۱۰۰۰، زنده‌مانی رده سلولی سرطان کولون CT-26 را به‌ترتیب ۲۴/۳۴، ۳۶/۰۰ و ۴۰/۰۸ درصد کاهش داد، در حالی که ترکیبات حاصل از آبکافت پروتئین‌های پرومود (Pro1، Pro2 و Pro3) باعث کاهش زنده‌مانی شدند به‌ترتیب ۲۶/۲۶، ۳۵/۹۱ و ۳۷/۱۳ درصد. Pep1 و Pro1 بالاترین اثرات سمیت سلولی را نشان دادند ($P < ۰/۰۵$). یافته‌های این مطالعه نشان می‌دهد که پپتیدهای زیست‌فعال موجود در *کلرلا ولگاریس* ممکن است شامل ترکیبات فراسودمند مفیدی برای پیشگیری از سرطان باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] Ultrafiltration جداسازی مواد خیلی ریز یا کلوئیدی از شاره به‌وسیلهٔ غشاهایی با منافذ بسیار ریز یا نیمه‌تراوا

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