Original Article

APPLIED FOOD BIOTECHNOLOGY, 2016, 3 (3):160-169
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Potential Health Effects of Enzymatic Protein Hydrolysates from *Chlorella vulgaris*

Mahsa sedighi[1], Hasan jalili[1], Seyed Omid Ranaei-Siadat[2], Abdeltif Amrane[3]*

1. Life Science Engineering Department, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran.
2. Nano-biotechnology Engineering Lab., Department of Biotechnology, Faculty of Energy Engineering and New Technologies, Shahid Beheshti University, Tehran, Iran.
3. Ecole Nationale Supérieure de Chimie de Rennes, Université de Rennes 1, CNRS, UMR 6226, 11 Allée de Beaulieu, CS 50837, 35708 Rennes Cedex 7, France.

Abstract

Background and Objective: *Chlorella vulgaris* is a multi-cellular edible algal species with abundant proteins. Extraction of high value protein fractions for pharmaceutical and nutritional applications can significantly increase the commercial value of microalga biomasses. There is no known report on the anticancer peptides derived from the *Chlorella vulgaris* abundant protein.

Materials and Methods: This study examined the antimicrobial and anticancer effects of peptides from a hydrolyzed *Chlorella vulgaris* protein with 62 kDa molecular weight. Protein hydrolysis was done by pepsin as a gastrointestinal protease, and was monitored through protein content measurement, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and high performance liquid chromatography measurements. Inhibitory effect of the produced peptides on *Escherichia coli* cells and breast cancer cell lines was assayed.

Results and Conclusion: Hydrolyzed peptides induced a decrease of about 34.1% in the growth of *Escherichia coli*, and the peptides of 3 to 5 kDa molecular weight had strong impact on the viability of breast cancer cells with IC_{50} value of 50 µg $µl^{-1}$. The peptide fractions demonstrating antimicrobial and anti-cancer activities have the potential for use as functional food ingredients for health benefits. These results demonstrate that inexpensive algae proteins could be a new alternative to produce anticancer peptides.

Conflict of interest: The authors declare that there is no conflict of interest.

Article Information

Article history
Received 1 Feb 2016
Revised 15 May 2016
Accepted 6 June 2016

Keywords
Bioactive peptides
Microalgae
Pepsin
Protein

Correspondence to
Hasan Jalili
Life Science Engineering Department, Faculty of New Sciences and Technologies
University of Tehran
Tehran, Iran.
P.O. Box: 1439957131
Tel: +98-21-61115775
Fax: +98-21-88617087
E-mail: hjalili@ut.ac.ir

1. Introduction

Microalgae with mostly as small as about 3-20 µm cells are from the primitive simple organized members of the plant kingdom. These algae are ubiquitous in nature to the extent that aquatic micro-algae have been found in the extreme conditions from hot springs to glacial ice flows. Being photo-synthetic and constituting the basis of the marine food chain, these microorganisms play a key role in the oceans' productivity [1]. In recent years micro-algae biotechnology has attracted an increasing interest for obtaining foods and biomolecules, com-prising proteins, vitamins, pigments, polyunsatur-ated fatty acids, and substances with pharmaceutical activities [2-4]. There is also a constant demand for proteinaceous food materials and single cell protein production [5]. Microalgae proteins are among the most favorable protein sources in foods due to their particular amino acids' composition necessary for numerous biological and physiological functions [6]. Since over 50% of most microalgae biomasses are composed of proteins, these macromolecules appear to play an important role in these green plants [7]. Because of their richness and amino acid profiles, microalgae proteins have been considered as alternative protein sources in foods [8,9], however, the food application of microalgal proteins

has not achieved significant importance because non-protein components lead to undesired changes in color, taste, and structure [10]. To increase potential food applications and commercial values, microalgae proteins have to be isolated with intact molecular structure and without any intense color and taste [11].

As there is a strong relationship between diet and health, food is a very vital factor for our health. Functional foods have positive physiological effects on health, and decrease the risk of diseases. Some natural biologically active ingredients (i.e. antioxidant, antiviral and antihypertensive), extracted from microalgae, are in great attention in food science and technology [12-15]. Microalgal extracts are crucial components of functional foods because they are considered as natural bioactive ingredients. In this regard, extracts from Chlorella species can be added to functional foods due to the antioxidant, antimicrobial, anti-inflammatory and antiviral properties of bioactive compounds [1,16].

Recently, cancer has turned into the largest single cause of death in the world, and chemoprevention has been a promising anticancer approach along with reducing the morbidity and mortality of cancer [17]. A variety of natural compounds have been shown to be beneficial for the inhibition of cancer, such as flavonoids, phenolic acids, carotenoids, etc. [18]. Peptides from the enzymatic hydrolysis of various food proteins exert quite different bioactivities; however, there are few publications on anticancer peptides. Some natural and synthetic peptides have been reported to show anticancer activities. A polysaccharide peptide extracted from mushroom showed antitumor activities. Dolostatins 10 and 15 are peptides isolated from marine sea hare *Dolabella auricularia* known to have antitumor activities on several cancer cell lines. Several Numerous antimicrobial peptides isolated from African frog, Streptomyces, and Micromonospora have both antimicrobial and anticancer activities [19]. Marine proteins enjoy wide sources and abundant content; therefore, activated peptides from marine food have been gaining attention in the past few years. The applications of microalgae are as nutritional supplements, natural dyes, and skin care products but there is no study reporting the anticancer activity of microalgae protein derived peptides.

C. vulgaris is an unicellular green algae found in both fresh and marine waters, and are widely used as a food supplement throughout the world [20]. The nutritional value of *C. vulgaris* was initially determined in the 1950s and 1960s [21]. This organism is a valuable source of nutrients with significant physiological and biochemical attributes, and has a relatively low cellulose content [22]. Various beneficial effects of *C. vulgaris* have been reported (e.g., prevention and treatment of cancer, as well as bacterial, fungal and viral infections) [23], promoting the growth rate of animals, raising immune functions, accelerating dioxin elimination, preventing the stress-included ulcers, greatly decreasing the high fat diet induced dyslipidemia, improvement of diabetic hyperglycemia, and anti-inflammation property [24]. Based on these biological properties, *C. vulgaris* is commonly used in nutrition and in pharmaceutical and biomedicine research projects [9,25]. However, the biomass of *C. vulgaris* has a low protein digestibility due to its strong cell wall [26]; therefore, there is a need for digestibility improvement through cell wall disruption in order to be used in human foods and drugs [27].

The enzymatic hydrolysis of microalgal cell proteins has been described as a promising method for the improvement of protein digestibility because most microalgae are indigestible to monogastric animals and humans [25,28-30]. However, to our knowledge, no report is available in the scientific literature on the biological activity of the major proteins of *C. vulgaris* or their biopeptide hydrolysates. For this reason, the objective of the present research was to optimize the intracellular extraction of *C. vulgaris* proteins, and then to evaluate the effect of pepsin-hydrolyzed peptides on *Escherichia coli* and breast cancer cell lines aiming to introduce them as functional food ingredients.

2. Materials and Methods

2.1. Materials

All chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Pepsin (EC number: 3.4.23.1. and ≥2500 units per mg dry weight activity) was purchased from Worthington Biochemical Co. (Lakewood, NJ, USA). All solutions, prepared with double-distilled water, were kept at 4°C before further use.

2.2. Microalgae, cultivation conditions and biomass processing

The unicellular green algae *C. vulgaris* 211/11B was kindly provided by the Agricultural Biotechnology Research Institute of Iran (ABRII), and was cultivated in 3N-BBM/vit medium (available at: http://www.ccap.ac.uk/). The inoculated flasks were incubated at 24°C under manual shaking and irradiated at 4000 Lux. The microalgae cells were collected at mid-log growth phase by centrifugation (1500 ×g at 15 min) and stored as pellets at -20°C.

2.3. Protein extraction and purification

Protein extraction started with suspending 5 g of frozen algal sample in 40 ml distilled water. The algal suspension was homogenized for 10 min in a mortar with liquid nitrogen using the following lysis buffer: 9 M urea, 4% w v^{-1} CHAPS (3-(3-cholamidopropyl) dim-ethyl-ammonio1propanesulfonate), 40 mM Tris–base, 100 mM DTT (dithiothreitol) and 0.1 M PMSF [31]. The homogenized mixture was centrifuged at 9000 ×g for 30 min to separate the cell debris. Total proteins

contained in the supernatants were then precipitated by 50% w v^{-1} ammonium sulfate.

Protein content determination was carried out using the Bradford method [32], which is a spectrophotometric determination at 595 nm using Coomassie Blue G-250 as a protein binding dye. Bovine serum albumin (BSA) was used as standard protein. The protein composition of the algal extracts was visualized using SDS-PAGE (Bio-Rad Laboratories, CA, USA) according to the instructions of the manufacturer. Samples were prepared with 1:1 ratio of the supernatants and SDS sample buffer and ran at 120 V for 90 min. Gels with 10% concentration were stained with Coomassie Brilliant Blue R-250.

HPLC analyses were carried out using HPLC-UV equipment (KNUER, Germany) equipped with a Polaris C18 cartridge column. The flow rate and injection volume were 1 ml min^{-1} and 50 µl, respectively. The protein samples were filtered through a 0.2 µm filter before the HPLC analysis. Analyses of proteinaceous materials were performed in a gradient procedure method with acetonitrile and water as eluent that started with 10% acetonitrile and reached to 90% in 30 min. To investigate probably bonded pigments with the extracted protein, Photodiode Array (PDA) detector was used in the range of 200 to 600 nm.

2.4. Preparation of hydrolysate peptides

Pepsin hydrolysates were prepared in simulated human gastric conditions as follows: the separated protein was digested by pepsin using an enzyme to substrate the ratio of 2% w w^{-1} at pH 2.0, at 37°C for 15 h. The digestion was adjusted to pH 7.0 and heated in a boiling water bath for 10 min at the end of the reaction to inactivate the enzyme. To remove the enzyme, the hydrolysates were centrifuged at 4000 ×g for 15 min and were then filtered through a membrane with a pore diameter of 0.2 µm.

The enzymatic hydrolysates obtained under the above mentioned conditions were ultrafiltered using Amicon 10, 5 and 3 kDa molecular weight cutoff (MWCO) membranes, respectively (Billerica, MA, USA). The fractions [smaller than 3 (<3) kDa, in the range of 3 to 5 (3–5) kDa, in the range of 5 to 10 (5-10) kDa, and higher than 10 (>10 kDa)] were collected, and their antimicrobial and antitumor activities were measured.

2.5. Determination of peptide concentration

Spectrophotometric assay using ophthaldialdehyde (OPA) was used for the determination of the proteolysis degree [33]. 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}), 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 of the samples together with 1 ml of OPA solution were added to the test tube and after 2 min, the reading with spectrophotometer was performed at 340 nm using distilled water as control.

2.6. Determination of antibacterial properties

Antibacterial activity of each protein fraction was investigated to select for the next stage of the experiments the fraction displaying the maximum antibacterial activity. Antibacterial activity of the peptide fractions was determined against *E. coli* CECT 434. For this purpose, *E. coli* was cultured in Liquid broth medium (10 g l^{-1} Tryptone, 10 g l^{-1} NaCl, 5 g l^{-1} Yeast Extract) for 16 h at 37°C. 50 µl of each fraction sample was added to 200 µl of the bacterial culture and incubated at 37°C with shaking. The inhibitory effect was measured at regular time intervals based on optical density at 600 nm.

2.7. Cell culture

Human breast adenocarcinoma cell lines (MCF-7 cell line) were seeded in appropriate culture medium (RPMI 1640, 10% FBS, 1% Pen/Strep). The cells were plated at a density of 10^4 cells/well in a 96-well microtiter plate overnight. Tissue culture plates were incubated at 37°C in humidified 5% CO_2 atmosphere.

2.8. MTT assay

Cytotoxic levels of the peptide fractions in the cultured MCF-7 breast cancer cells were measured using [3-(4,5-dimethyl thiazole-2-yl)]c-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich)-based cell titer assay [34]. The cells were allowed to attach and grow for 36 h. The old medium was then replaced with a fresh medium and treated with varying concentrations of peptide fractions (0-160 µg ml^{-1}). Each sample was added into the wells to determine the effect on growth according to the time and concentration. MTT dye was added after each time-point followed by the determination of the formation of colored formazan product by a detergent solution. The plate was read at 570 nm in a TECAN Micro plate Reader (Infinite® 200 PRO series, Männedorf, Switzerland). For control, appropriate row or column of wells was left untreated at each time point. The hydrolysate concentration, which gives 50% growth inhibition, is referred to as the IC_{50}. A simple method for calculation of the IC_{50} (Eq. 1) is performed by linear interpolation between the concentrations above and beneath 50% inhibition in the dose response curve (= two flanking points) that is calculated as follows:

$$\text{Formula} = \text{EXP}\left(\ln(\text{conc} > 50\,\%) - \left(\frac{(\text{signal} > 50\% - 50)}{(\text{signal} > 50\% - \text{signal} < 50\%)} \times \ln\frac{\text{conc} > 50\%}{\text{conc} < 50\%}\right)\right) \quad \text{Eq. 1}$$

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

2.9. Statistical analysis

Experimental data were analyzed using the SPSS16.0 software (SPSS, Chicago, IL, USA) with sample means and standard errors of 10%. All tests were conducted in triplicate. Experimental data were expressed as mean±standard deviation.

3. Results and Discussion

3.3. Optimum protein extraction method

Culturing of microalgae was carried out by autotrophic outdoor cultivation of *C. vulgaris* at room temperature with shaking system. The high protein content (>50% of biomass) of *C. vulgaris* was one of the reasons for selecting this organism as an unconventional protein source. To extract the whole protein, several methods were examined, including homogenization, sonication and glass beads for cell wall disruption and achieving maximum number of protein fractions extraction. According to the protein concentration, as well as the SDS-PAGE and HPLC results, among the tested methods, maximum protein fractions were obtained with liquid nitrogen and lysis buffer extraction method (data not shown).

Among the protein fractions obtained, the fraction with 62 kDa molecular weight had the highest concentration. Figure 1 shows the SDS-PAGE gel (Fig. 1a) and HPLC chromatogram (Fig. 1b) of the selected protein bonds among several proteins. The HPLC chromatogram confirmed the above results, and scanning in the 200-600 nm wave length range demonstrated that no pigment was bonded to the protein (Fig. 1c). Therefore, this extraction method proved to be a rapid, simple and efficient approach to isolate the highest concentration of *C. vulgaris* protein fractions.

3.4. Enzymatic hydrolysis of the extracted protein

In order to simulate human gastric conditions, this study utilized pepsin for protein hydrolysis at pH 2.0 and a temperature of 37°C for 15 h of digestion time. This experiment is based on 62 kDa protein, the most abundant protein fraction in the whole protein profile. This hydrolysate had peptides of various size ranges, differentiated as follows: <3 kDa, 3-5 kDa, 5-10 kDa and >10 kDa, and their impact on cell viability was determined. The degree of hydrolysis measured by the OPA method was increased to 2000 µg ml^{-1} for all fractions. Optimal concentration of the efficient peptide fractions was determined by examining the impact on cells of each fraction at concentrations ranging from 0 to 160 µg ml^{-1}.

3.5. Cytotoxic activity of the hydrolyzed peptide fractions

High sensitivity and stability are among the characteristics that have made anticancer and antimicrobial peptides very interesting topics for researchers. Animal and plant food proteins have been regarded as main sources of bioactive peptides [35]. Generating bioactive peptides from foods has been the subject of many researches in recent years [36]. Some marine fish species like salmon, oyster, macroalgae, squid, sea urchin, shrimp, snow crab, and seahorse have also been used as sources of bioactive peptides [35]. Based on previous studies, potent biological activities (i.e., antihypertensive, antioxidant, immunomodulatory, anticancer, antimicrobial, and lipid lowering activities) have been shown by protein hydrolysates.

The selected protein was hydrolyzed with pepsin under gastric medium conditions, and the hydrolysed peptides were separated according to their molecular weight as follows: <3, 3–5, 5–10 and >10 kDa. To meet the aim of this study, the antibacterial properties of peptides were investigated on *E. coli* and the results were also compared to the antibacterial properties of the whole *C. vulgaris* biomass and 62 kDa protein. As shown in Fig. 2, the hydrolysed peptides have the highest inhibitory effect of 34.1% on *E. coli* following 16 h of incubation. It is worth noting that both the *C. vulgaris* biomass and the 62 kDa protein inhibited bacterial growth to a certain extent. The comparison between proteins and their hydrolysate peptides suggests the significant role of bioactive peptides, which can be, therefore, considered as latent factors in parent proteins before hydrolysis. The percentages of inhibition, shown in Table 1, indicate that the effect of hydrolysate peptides was 8.5 and 1.6 times greater than that of the Chlorella biomass and its proteins, respectively, suggesting that the peptides effectively induced the cell wall destruction of *E. coli* and inhibited the growth of cells.

Table 1. Inhibitory effect of *C. vulgaris* biomass, its purified proteins and produced bioactive peptides on *E. coli*

Test material	Inhibitory effect (%)
Control*	0
Chlorella biomass	4.0
Proteins	21.2
Bioactive peptides	34.1

* *E. coli* grown in pure culture medium

Antimicrobial assay between the extracted protein and the peptides was done without fractionation because it was necessary to make a distinction between these two samples for their potential of growth inhibition, and to find out if there are some peptides in parent protein that act just with hydrolysis. Actually, it was an initial test to make sure if the produced peptides have a nutraceutical effect.

Because of the side effects of cancer treatment, alternative procedures such as the use of antimicrobial peptides are investigated. Some studies have shown antimicrobial peptides to be cytotoxic for cancer cells and to be able to prevent their undesired and uncontrolled growth [37]; for this reason, antimicrobial peptides are regarded as new antibiotics

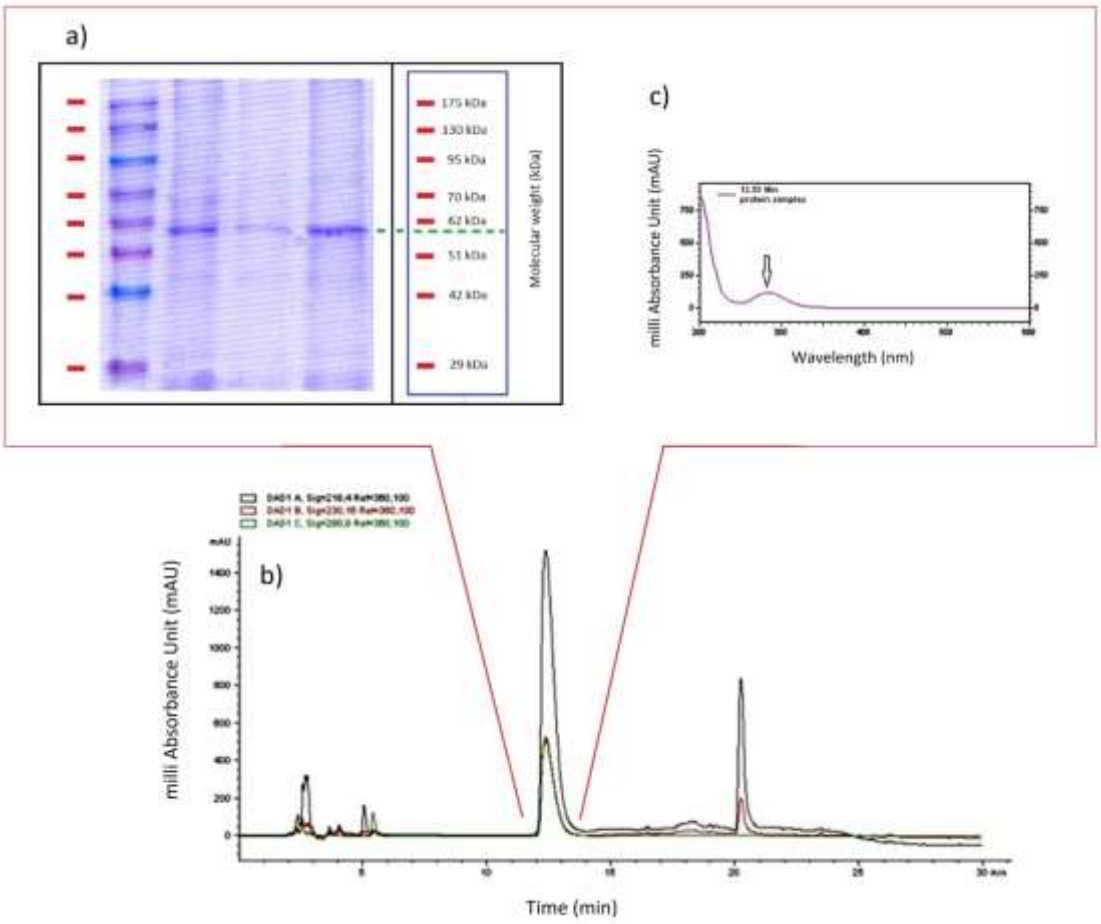

Figure 1. Analysis of the extracted protein fraction: a) SDS-PAGE profile of selected protein inside the ladder sample, b) HPLC chromatogram in 218 (black), 230 (red) and 280 (green) nm, and c) scanning in the 200-600 nm wavelength range to assess lack of pigments.

having strong antimicrobial properties, and are administered against antibiotic resistant bacteria [38].

To compare the anticancer properties of peptide fractions with those of the *C. vulgaris* biomass and the 62 kDa protein, the effect of all samples on MCF-7 cell line at a given concentration of 2 μg μl^{-1} was examined for 24, 48 and 72 h. As shown in Fig. 3, the results indicated a dose- and time-dependent inhibition in the growth of MCF-7 cells. At a concentration of 160 μg μl^{-1}, the 3-5 KDa peptide fractions showed the highest inhibitory effect on MCF-7 cells, with a cell growth reduction of over 60% after 72 h of incubation. As it is obvious in Fig. 3, there is a significant difference between the biomass effect and the peptide fractions on the cells. However, the inhibitory effect of the parent protein had little difference with the peptide fractions due to the existence of peptides in the extracted protein sequences with less effect than released peptides. So, to reach more inhibition on the growth of cancer cells, it was necessary to hydrolyze the extracted protein and find the efficient peptide fractions IC_{50} is a good measure of the inhibitory effect of drugs. As shown in Figures 3 and 4, the 3-5 kDa bioactive peptides (50 μg μl^{-1}) were capable of reducing cell viability by 50% after 72 h. In contrast, treatments with the 3-5 kDa fractions at 24 h time-point revealed negligible inhibition of the growth of MCF-7 cells compared to the untreated cells (Figure 3). For 48 h time-point, the inhibitory effect was pronounced, even if the IC_{50} was not reached. It was also observed that both 48 and 72 h time-points showed similar values at concentrations below 10 μg μl^{-1} whereas higher decreases were observed for the 72 h time-point at concentrations above 10 μg μl^{-1}.

Wang et al. reported that enzymatic hydrolysates from different soy varieties inhibited the viability of cultured leukemia cells with the IC_{50} values of 3.5 to 6.2 mg ml^{-1}, which were significantly lower than the activity of lunasin (a well-known protein derived peptide in soy) with the IC_{50} value of 0.078 mg ml^{-1} [39]. Recent studies have shown that low molecular size peptides from Pacific oyster hydrolysates have induced the dose-dependent growth inhibition of transplanted murine sarcoma in BALB/c mice possibly via increased immune stimulation [35].

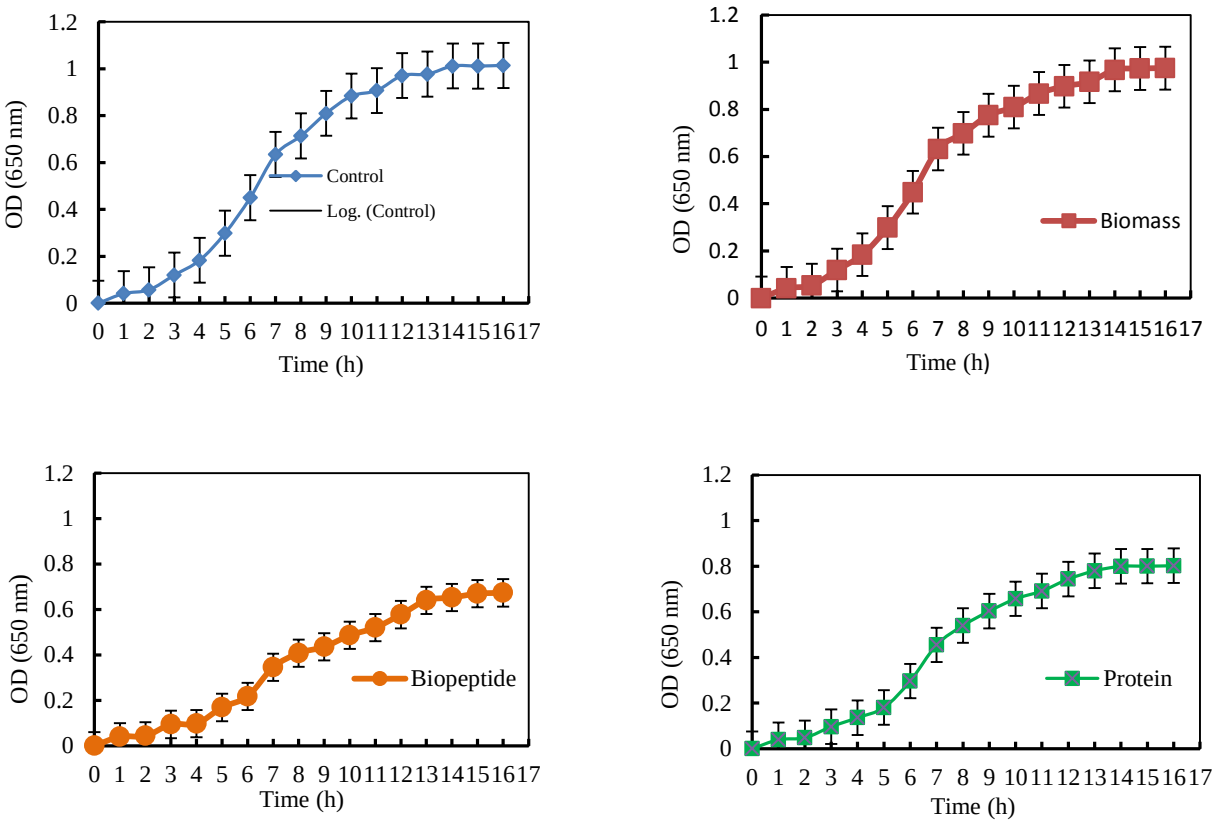

Figure 2. *E. coli* growth curve for determination of the antimicrobial activity of *C. vulgaris* biomass, 62 kDa protein and bioactive peptides compared to a control culture (all experiments were carried out in triplicate).

Diverse molecules with pharmacological potential are found in plants. For example, thonins that are small cysteine rich peptides (˜5 kDa) with toxic and antimicrobial properties were the first AMP isolated from plants. Cytotoxic and anticancer activities are shown by several plant thionins, e.g., pyrularia thionin from mistletoe (*Pyrularia pubera*) with an IC_{50} of 50 µg $µl^{-1}$ against the cervical cancer cells (HeLa) and mouse melanoma cells (B16). Poor stability and susceptibility to proteolytic digestion are some of the problems that must be solved in future [40]. Colon and breast anticancer effects of peptide hydrolysates derived from rice bran were shown by Kannan et al. With an IC_{50} of 770 µg $µl^{-1}$, the <5 kDa peptide fraction separated from rice bran protein hydrolysate demonstrated a potent antitumor activity against cancer cells [41].

However, few publications have studied the anticancer activities of marine proteins. Significant growth inhibitors on MCF-7/6 and MDA-MB-231 cell lines were found to be from three blue whiting, three cod, three plaice and one salmon hydrolysate. They contained a complex mixture of free amino acids and peptides of various sizes ranging up to 7 kDa, based on the composition analysis [42]. In order to produce bioactive hydrolysates, seven commercial proteases (Protamex, Trypsin, Neutrase, Savinase, NS37005, Esperase and Alcalase) were also used to hydrolyze gelatin obtained from giant squid (*Dosidicus gigas*) inner and outer tunics. The most potent angiotensin-converting enzyme inhibitor was Alcalase hydrolysate (IC_{50} = 0.34 mg ml^{-1}) while the highest cytotoxic effect on cancer cells was shown by Esperase hydrolysate with the IC_{50} values of 0.13 and 0.10 mg ml^{-1} for MCF-7 and U87 (glioma) cell lines, respectively [43].

Recently, great interest has been shown for microalgal proteins as sources of bioactive peptides because of their therapeutic potential in the treatment of various diseases. Peptides are released from the parent proteins by the action of an enzyme (usually a protease); when consumed as pure peptides or hydrolysates, they can exert their function by modulating the structure and function of metabolic enzymes, implicated in certain diseases, after absorption into the blood circulatory system. In this research, *C. vulgaris* was introduced as a source of bioactive peptides to be used as functional food ingredients or pharmaceutical products with antibacterial and anticancer effects.

Several pharmaceutical actions of enzymatic protein hydrolysates from *C. vulgaris* have been investigated, and the inhibition of angiotensin-converting enzyme, the antioxidant functionality and the hypocholesterolemic effects have been the most studied. Additionally, different peptides from various sources exhibit an anticancer activity; therefore, they can be used to the prevention or treatment

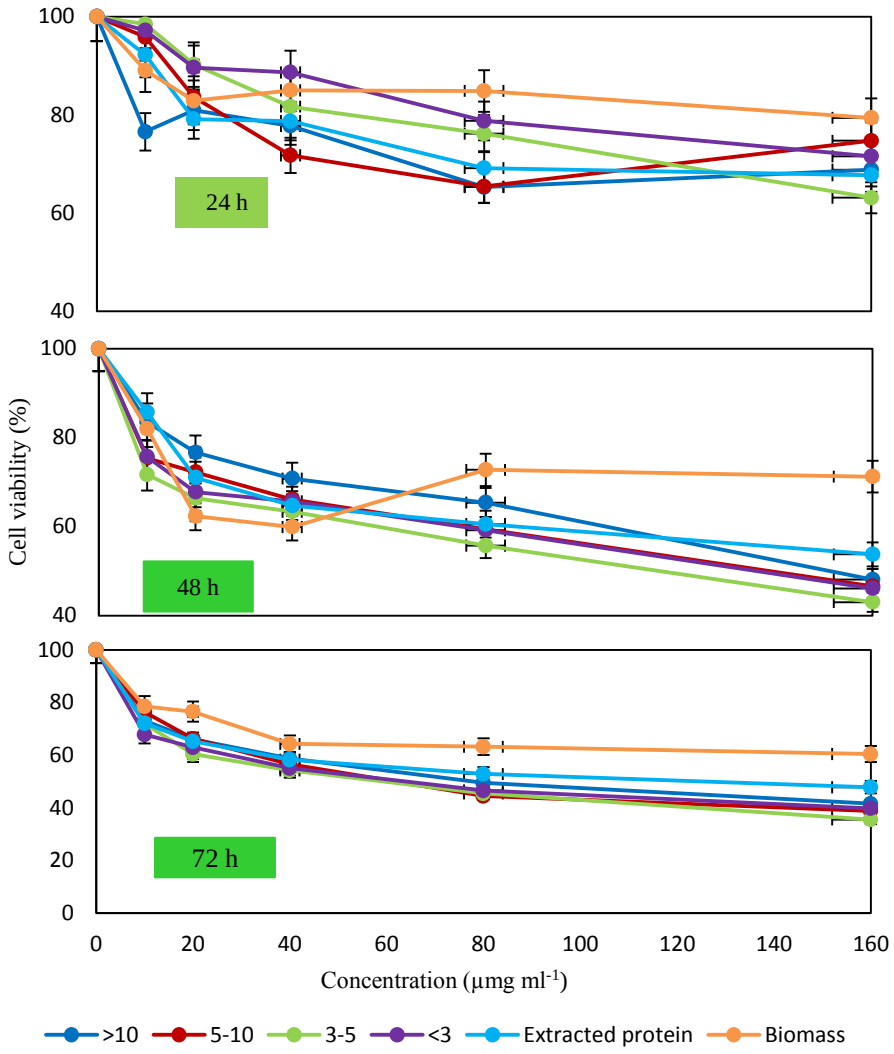

Figure 3. Comparison of various samples (i.e., *C. vulgaris* biomass, extracted protein and produced bioactive peptides) at different concentrations for their anti-proliferative effect on MCF-7 cell line (all experiments carried out in triplicate).

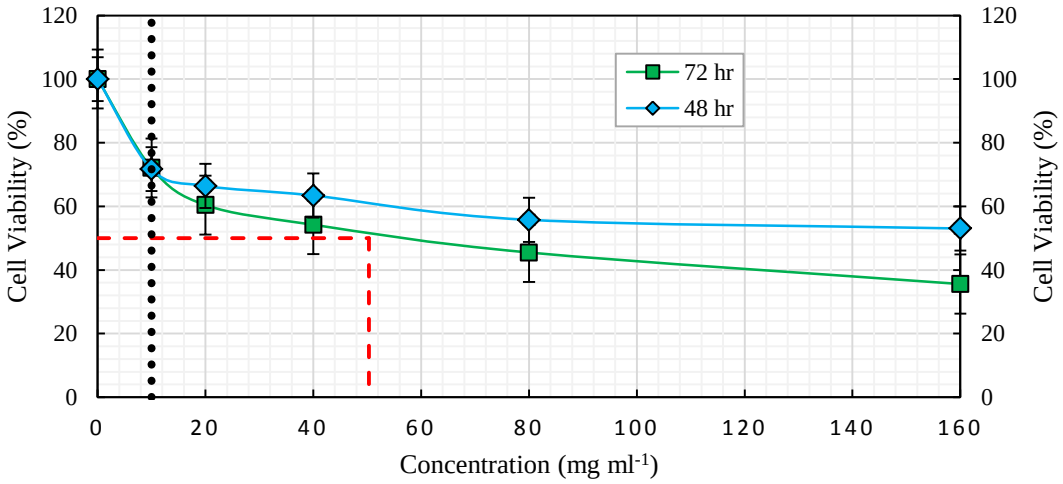

Figure 4. MTT assay for determination of the anti-proliferative activity of 3-5 kDa fractions at 48 and 72 h time-points with MCF-7 cells. Red line shows peptide concentration with 50% inhibition activity (IC50) for 72 h, and black line characterizes the constant area for 48 and 72 h time-points (all experiments carried out in triplicate).

of cancer through nutritional interventions. Sheih et al. reported the anticancer and antioxidant activities of peptides from *C. vulgaris* protein waste [22,25]. In their research, the peptides were isolated from the pepsin hydrolysate of algal protein waste, and among these peptides, a potent anti-proliferative, antioxidant and NO-production inhibiting peptide was introduced. They studied the anticancer effect of *C. vulgaris* protein waste hydrolysates on the AGS cell line in which the peptide fraction showed a dose dependent growth inhibitory effect on the AGS cell line with the IC50 value of 70.7±1.2 μg ml^{-1}.

4. Conclusion

In the present study, a protein extracted from *C. vulgaris* with biological activity and without an intense color and taste was chosen. Because the absorption span of microalgae pigments is in the visible spectrum, the absorption of the effective protein fraction was evaluated in the wavelength range of 200-600 nm, and no peak was observed. This finding appears to be helpful to ascribe anticancer activity to proteins without any additional materials. Finally, the protein extracted was cleaved to bioactive peptides through proteolysis by pepsin. Enzymatic hydrolysis of the proteins was done by pepsin in simulated human gastric media. Since the literature dealing with the digestibility of *C. vulgaris* proteins remains scarce, it was necessary to investigate and characterize hydrolysis of the selected protein. To simulate the gastric conditions, pepsin was used for protein hydrolysis. Some microalgae have shown potent cytotoxic activities, and polysaccharides and terpenoids are considered as contributing factors for anticancer effects among those. To the best of our knowledge, there has been no study on anticancer peptides from microalgae protein, and this is the first report on MCF-7 cell inhibition by peptides from *C. vulgaris* abundant protein.

The intensification in the production of functional foods supplemented with bioactive molecules such as carbohydrates, lipids, proteins and carotenoids produced by microalgae is a consequence of the growing need for people to improve their nutritional status because of the rhythm of modern life. As shown in this study, algal soluble proteins were extracted from *C. vulgaris* in an effective and efficient way to produce food ingredients. The results suggest that enzymatic protein hydrolysates from *C. vulgaris* could reduce cancer cell viability. In recent years, microalgae have been introduced as a functional food. Our study indicated that at least one protein in *C. vulgaris* produces hydrolysate peptides with antibacterial and anticancer functionality under gastric conditions by pepsin hydrolysis. The peptide fractions that demonstrate anticancer activities have the potential for use as functional food ingredients for health benefits. However, preparing cancer therapeutics is limited to ‘‘proof of concept’’ studies, and a long road lies ahead to the actual use of these biomaterials in clinical practice.

5. Acknowledgments

This work was supported by the NanoBiotechnology Engineering Laboratory in Shahid Beheshti University (Tehran, Iran).

6. Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Chojnacka K, Kim SK. Introduction of Marine Algae Extracts, in Marine Algae Extracts. Wiley VCH Verlag GmbH & Co. KGaA. 2015; 1-14.
2. El Gamal AA. Biological importance of marine algae. Saudi Pharm J. 2010; 18(1): 1-25. doi: 10.1016/j.jsps.2009.12.001.
3. Pulz O, Gross W. Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol. 2004; 65(6): 635-648. doi: 10.1007/s00253-004-1647-x
4. Morris HJ, Carrillo OV, Almarales A, Bermudez RC, Alonso ME, Borges L, Quintana MM, Fontaine R, Liaurado G, Hernandez M. Protein hydrolysates from the alga *Chlorella vulgaris* 87/1 with potentialities in immunonutrition. Biotecnol Apl. 2009; 26(2): 162-165.
5. Anupama, Ravindra P. Value-added food: Single cell protein. Biotechnol Adv. 2000; 18(6): 459-479. doi: 10.1016/S0734-9750(00)00045-8
6. Zhang B, Zhang X. Separation and nanoencapsulation of antitumor polypeptide from *Spirulina platensis*. Biotechnol Prog. 2013; 29(5): 1230-1238. doi: 10.1111/j.1750-3841.2011.02455.x
7. Walker T.L, Purton S, Becker D.K, Collet C. Microalgae as bioreactors. Plant Cell Reports, 2005; 24(11): 629-641. doi: 10.1007/s00299-005-0004-6.
8. Becker E.W. Micro-algae as a source of protein. Biotechnol Adv. 2007; 25(2): 207-210. doi: 10.1016/j.biotechadv.2006.11.002.
9. Plaza M, Herrero M, Cifuentes A, Ibanez E. Innovative natural functional ingredients from microalgae. J Agric Food Chem. 2009; 57(16): 7159-7170. doi: 10.1021/jf901070g.
10. Kim SK, Wijesekara I. Development and biological activities of marine-derived bioactive peptides: A review. J Funct Foods. 2010; 2(1): 1-9. doi: 10.1016/j.jff.2010.01.003.
11. Schwenzfeier A, Wierenga PA, Gruppen H. Isolation and characterization of soluble protein from the green microalgae *Tetraselmis sp.* Bioresour Technol. 2011; 102(19): 9121-9127. doi: 10.1016/j.biorthch.2011.07.046.
12. Buono S, Langellotti AL, Martello A, Rinna F, Fogliano V. Functional ingredients from microalgae. Food Funct. 2014; 5(8): 1669-1685. doi: 10.1039/c4fo00125g.

13. Ibañez E, Cifuentes A. Benefits of using algae as natural sources of functional ingredients. J Sci Food Agric. 2013; 93(4): 703-709.

14. Lordan S, Ross RP, Stanton C. Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. Mar Drugs. 2011; 9(6): 1056-1100. doi: 10.3390/md9061056.

15. Plaza M, Cifuentes A, Ibáñez E. In the search of new functional food ingredients from algae. Trends Food Sci Technol. 2008; 19(1): 31-39. doi: 10.1016/j.tifs.2007.07.012.

16. Kim SK, Chojnacka K. Marine Algae Extracts: Processes, Products, and Applications, 2 Volume Set. 2015: John Wiley & Sons. ISBN 978-3-527-33708-8.

17. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Exp Cell Res. 2000; 256(1): 42-49. doi: 10.1006/excr.2000.4838.

18. Russo M, Tedesco I, Iacomino G, Palumbo R, Galano G, Russo GL. Dietary phytochemicals in chemoprevention of cancer. Current Med Chem-Immun, Endoc & Metab. Agents. 2005; 5(1): 61-72. doi: 10.2174/1568013053005454.

19. Kim SE, Kim HH, Kim JY, Kang YI, Woo HJ, Lee HJ. Anticancer activity of hydrophobic peptides from soy proteins. Biofactors. 2000; 12(1-4): 151-155. doi: 10.1002/biof.5520120124.

20. Kralovec JA, Power MR, Liu F, Maydanski E, Ewart HS, Watson LV, Barrow CJ, Lin TJ. An aqueous *Chlorella* extract inhibits IL-5 production by mast cells in vitro and reduces ovalbumin-induced eosinophil infiltration in the airway in mice in vivo. Int Immunopharmacol. 2005; 5(4): 689-698. doi: 10.1016/j.intimp.2004.11.016.

21. Janczyk P, Franke H, Souffrant WB. Nutritional value of *Chlorella vulgaris*: Effects of ultrasonication and electroporation on digestibility in rats. Anim. Feed Sci Technol. 2007; 132(1–2): 163-169. DOI: 10.1016/j.anifeedsci.2006.03.007.

22. Sheih IC, Fang TJ, Wu TK, Lin PH. Anticancer and antioxidant activities of the peptide fraction from algae protein waste. J Agric Food Chem. 2009; 58(2): 1202-1207. doi: 10.1021/jf903089m.

23. Smith VJ, Desbois AP, Dyrynda EA. Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. Mar Drugs. 2010; 8(4): 1213-1262. doi: 10.3390/md8041213.

24. Chen CL, Liou SF, Chen SJ, Shih MF. Protective effects of *Chlorella*-derived peptide on UVB-induced production of MMP-1 and degradation of procollagen genes in human skin fibroblasts. Regul Toxicol Pharmacol. 2011; 60(1): 112-119. doi: 10.1016/j.yrtph.2011.03.001.

25. Sheih IC, Wu TK, Fang TJ. Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. Bioresour Technol. 2009; 100(13): 3419-3425. doi: 10.1016/j.biortech.2009.02.014.

26. Mendez L, Mahdy A, Ballesteros M, Gonzalez-Fernandez C. *Chlorella vulgaris* vs cyanobacterial biomasses: Comparison in terms of biomass productivity and biogas yield. Energy Convers Manage. 2015; 92: 137-142. doi: 10.1016/j.enconman.2014.11.050.

27. Morris HJ, Almarales A, Carrillo O, Bermudez RC. Utilisation of *Chlorella vulgaris* cell biomass for the production of enzymatic protein hydrolysates. Bioresour Technol. 2008; 99(16): 7723-7729. doi: 10.1016/j.biortech.2008.01.080.

28. Kang KH, Qian ZJ, Ryu B, Kim D, Kim SK. Protective effects of protein hydrolysate from marine microalgae *Navicula incerta* on ethanol-induced toxicity in HepG2/CYP2E1 cells. Food Chem. 2012; 132(2): 677-685. doi: 10.1016/j.foodchem.2011.10.031.

29. Korhonen H, Pihlanto A. Bioactive peptides: production and functionality. Int Dairy J. 2006; 16(9): 945-960. doi: 10.1016/j.idairyj.2005.10.012.

30. Morris HJ, Carrillo O, Almarales A, Bermudez RC, Lebeque Y, Fontaine R, Liaurado G, Beltran Y. Immunostimulant activity of an enzymatic protein hydrolysate from green microalga *Chlorella vulgaris* on undernourished mice. Enzyme Microb Technol. 2007; 40(3): 456-460. doi: 10.1016/j.enzmictec.2006.07.021.

31. Tran NP, Park JK, Lee CG. Proteomics analysis of proteins in green alga *Haematococcus lacustris* (Chlorophyceae) expressed under combined stress of nitrogen starvation and high irradiance. Enzyme Microb Technol. 2009; 45(4): 241-246. doi: 10.1016/j.enzmictec.2009.07.006

32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72(1): 248-254. doi: 10.1016/0003-2697(76)90527-3.

33. Church FC, Swaisgood HE, Porter DH, Catignani GL. Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci. 1983; 66(6): 1219-1227. doi: 10.3168/jds.S0022-0302(83)81926-2.

34. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986; 89(2): 271-277. doi: 10.1016/0022-17598690368-6.

35. Udenigwe CC, Aluko RE. Food protein-derived bioactive peptides: Production, processing, and potential health benefits. J Food Sci. 2012; 77(1): R11-R24. doi: 10.1111/j.1750-3841.2011.02455.x.

36. Di Bernardini R, Harnedy P, Bolton D, Kerry J, O'Neill E, Mullen AM, Hayes M. Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. Food Chem. 2011; 124(4): 1296-1307. doi: 10.1016/j.foodchem.2010.07.004.

37. Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta, Biomembr. 2008; 1778(2): 357-375. doi: 10.1016/j.bbamem.2007.11.008.

38. Moghaddam MM, Aghamollaei H, Kooshki H, Azizi K, Mirnejad R, Choopani A. The development of antimicrobial peptides as an approach to prevention of antibiotic resistance. Rev Med Microbiol. 2015; 26(3): 98-110. doi: 10.1097/MRM.0000000000000032.

39. Wang W, Bringe NA, Berhow MA, Gonzalez de Mejia E. β-Conglycinins among sources of bioactives

in hydrolysates of different soybean varieties that inhibit leukemia cells in vitro. J Agric Food Chem. 2008; 56(11): 4012-4020. doi: 10.1021/jf8002009.

40. Guzm-Rodriguez JJ, Ochoa-Zarzosa A, Lopez-Gomez R, Lopez-Meza JE. Plant antimicrobial peptides as potential anticancer agents. BioMed Res Int. 2015; 2015: 1-11. doi: 10.1155/2015/735087.
41. Kannan A, Hettiarachchy N, Narayan S. Colon and breast anti-cancer effects of peptide hydrolysates derived from rice bran. Open Bioact Compd J. 2009; 4: 17-20.
42. Suarez-Jimenez GM, Burgos-Hernandez A, Ezquerra-Brauer JM. Bioactive peptides and depsipeptides with anticancer potential: Sources from marine animals. Mar Drugs. 2012; 10(5): 963-986. doi: 10.3390/md10050963.
43. Alemán A, Perez-Santin E, Bordenave-Juchereau S, Arnaudin I, Gomez-Guillen MC, Montero P. Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity. Food Res Int. 2011; 44(4): 1044-1051. doi: 10.1016/j.foodres.2011.03.010.
44. Mayer AM, Gustafson KR. Marine pharmacology in 2003-2004: anti-tumour and cytotoxic compounds. Eur J Cancer. 2006; 42(14): 2241-2270. doi: 10.1016/j.ejca.2006.05.019.