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Isolation of Bioactive Peptides from Rocky Shore Crab, Grapsus Albolineatus, Protein Hydrolysate with Cytotoxic Activity against 4T1 Cell Line

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ABSTRACT

Aims Breast cancer is the most common cancer in women in several countries. Bioactive peptides have demonstrated their cytotoxic potential in numerous cancer cell lines. In the search for novel bioactive peptides for pharmacological properties, crab is noncommercial protein-rich species. Using enzymatic hydrolysis is an efficient way to recover potent bioactive peptides from marine sources.

Materials & Methods The aim of this study was to isolate fractions from rocky shore crab hydrolysate with desired molecular weight by ultrafiltration and investigate their cytotoxic activities. Four fractions (>30kDa, 10-30kDa, 3-10kDa and <3kDa) were evaluated for cytotoxic activity against a 4T1 cell line by MTT assay.

Findings The MTT assay showed that although all fractions from the crab hydrolysate showed some activity, the low molecular weight samples (3-10kDa and <3kDa) were more effective than high molecular weight fractions (>30kDa and 10-30kDa) while the 3-10kDa fraction proved to be the most effective. The low molecular weight fractions significantly reduced the viability of the 4T1 cell lines in a dose-dependent manner upon 24 and 48h. The results were recorded in IC50 values of about 0.40±0.063mg mL-1 for <3 and 0.25±0.026mg mL-1 for 3-10kDa fractions.

Conclusion Peptide fractions were isolated from the protein hydrolysate of the rocky shore crab Grapsus albolineatus are able to inhibit cancer cells and can be considered as a novel agent in nutraceutical and pharmaceutical ingredient applications.

Keywords Rocky Shore Crab; Bioactive Peptide; Cytotoxic Activity; 4T1 Cell line

CITATION LINKS

[1] Bioactive peptides and depsipeptides with anticancer potential: Sources from marine ... [2] Anticancer activity of an oligopeptide isolated from hydrolysates of Sepia ... [3] Antioxidants, mechanisms, and recovery by membrane ... [4] Membrane bioreactors: Enzyme processes. Biotechnol Food Process ... [5] Antitumor peptides from marine ... [6] Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (Tegillarca granosa) ... [7] PLGA-loaded gold-nanoparticles precipitated with quercetin downregulate HDAC-Akt activities controlling proliferation and activate p53-ROS crosstalk to induce apoptosis in hepatocarcinoma ... [8] The bcl-2 and p53 oncoproteins can be modulated by bryostatin 1 and dolastatins in human diffuse large cell ... [9] Sepia ink oligopeptide induces apoptosis in prostate cancer cell lines via caspase-3 activation and elevation of Bax/Bcl-2 ... [10] In vitro and in vivo anticancer activity of pardaxin against proliferation and growth of oral squamous cell ... [11] Pore structure and synergy in antimicrobial peptides ... [12] Measurement of protein using bicinchoninic ... [13] Antiproliferative activity of fish protein hydrolysates on human breast cancer ... [14] Purification and identification of antioxidative peptides from loach (Misgurnus anguillicaudatus) protein hydrolysate ... [15] Purification, characterisation and stability of an antioxidant ... [16] Fish protein hydrolysates: production, biochemical ... [17] Effect of degree of hydrolysis on the antioxidant activity ... [18] Antimicrobial capacity and antioxidant activity of enzymatic ... [19] Anticancer and antioxidant activities of the peptide fraction ... [20] Anticancer activity of a hexapeptide from skate ... [21] Antioxidant and anticancer activities of enzymatic hydrolysates ... [22] Anticancer potential of bioactive peptides from animal ... [23] Release of multifunctional peptides by gastrointestinal digestion of sea ... [24] Isolation and identification of an antiproliferative ... [25]Antioxidant and chelating activity of Jatropha ... [26] Product inhibition in native-state prepared ... [27] Antioxidant activities of hydrolysates of Arca subcrenata ... [28] Recovery of Bioactive Peptide Fractions from Rainbow Trout (Oncorhynchus mykiss)Processing ...

Introduction

Enzymatic hydrolysis is considered as an efficient way to recover potent bioactive peptides from marine protein sources because several peptides obtained by this process have different bioactivities [1]. It is reported that these peptides usually contain 2-20 amino acid residues and their activity were highly influenced by their molecular weight and amino acid sequence, which are affected by different organism sources, and different approaches for isolation [2, 3]. Membrane ultrafiltration technology represents an attractive means to fractionate bioactive protein hydrolysate. Advantage major benefit of such a system is that the molecular weight distribution of the desired peptide can be controlled to a great extent by the adoption of ultrafiltration membrane [4]. Furthermore, the use of specific enzymes enables the selection of rupture sites in the protein sequence that could be determinant for peptide bioactivity.

Nature remains the largest source of bioactive peptides, whereas marine-derived bioactive peptides are considered as the prominent candidates with a novel mechanism for cancer prevention and treatment while they can directly kill cancer cells or induce cell apoptosis [5, 6].

Apoptosis is a programmed cell death by which cells that are no longer useful are directed to their deaths under normal circumstances. Increase in Reactive Oxygen Species (ROS) production during oxidative stress causes proliferation/differentiation, senescence, exhaustion, and consequently apoptosis in normal cells. In normal cells, several tumor suppressor genes, such as *p53*, *FoxO*, retinoblastoma (*RB*), *p21*, *p16*, and breast cancer susceptibility genes 1 and 2, modulate cells faced with oxidative stress to adapt to the remodeled redox balance, thus preventing lipid peroxidation and oxidative damage to DNA and protein. However, in the absence of wild type suppressor genes, especially *p53*, cancer cells shut down the emergency role of several antioxidative pathways in case of ROS accumulation [7].

Identification of caspase activators or tumor suppressor genes becomes one of apoptotic approach for the discovery of novel anticancer agents. In this regard, decreasing in Bcl-2 level with associating an increase in p53 expression in the lymphoma cell line could be apoptosis mechanism of dolastatin 10, a peptide isolated from a marine shell-less mollusk [8]. QPK a purified peptide from sepia ink hydrolysate, inhibited the proliferation of DU-145, PC-3, and LNCaP cells by decreasing the expression of the anti-apoptotic protein Bcl-2 and increasing the expression of the apoptogenic protein Bax and activating caspase-3 [9].

Previous researches also suggested that some of the anticancer peptides do not activate apoptosis. These peptides selectively target negatively charged membrane components in the membrane of cancer cells, such as phosphatidylserine, thus resulting in cytoplasmic membrane depolarization and cell death. Pardaxin a peptide from the Red Sea sole [10] and magainin a peptide from frogs [11], induce cell lysis via pore formation and follow this model.

Today, cancer has been a major killer of human beings. Therefore, identifying novel natural products with anticancer properties are needed to reduce the mortality of cancer. Rocky shore crabs live near the surface of the water column or in intertidal areas and exposed to UV light during their entire lives. Hence it seems that these organisms have modified adaptation strategies such as producing secondary metabolites, which enable them to survive in high-pressure environments. Considering this and the lack of information on Persian Gulf rocky shore crab *Grapsus albolineatus* protein hydrolysate bioactivity, we conducted this study to produce different molecular weight peptides from crab hydrolysate and investigate the cytotoxic effect of them on 4T1 cells.

Materials and Methods

Sample preparation: Fresh rock crab (*Grapsus albolineatus*) from the Persian Gulf (n: 15, mean of weight was equal to 55.00±2.63g) were cached from the rocky shore of Qeshm island in the summer 2017. The samples were immediately frozen at -20°C, and then the frozen crab transported in a Styropor box containing ice to the lab by airplane within 2h.

Preparation of enzymatic hydrolysate from crab: Fifty grams of minced crab (Whole body) preincubated at 85°C for 20min. Thereafter the complex was mixed with 100ml deionized water and homogenized prior to enzymatic hydrolysis. The hydrolysis reaction was started by the addition of alkalase (2.4L; 2.64AU/g; Sigma-Aldrich; USA) in the optimal conditions

(55°C and pH of 8.5) at an enzyme/substrate ratio of 2% (v/w) for 3h. After hydrolysis, the hydrolysates were heated in a boiling water bath (95°C) for 15min to inactivate the enzymes. Hydrolysate was centrifuged at 8000g for 10min.

Fractionation of crab hydrolysate by membrane ultrafiltration: For the fractionation of hydrolysate, the crab hydrolysate was sequentially fractionated based on molecular weight, using centrifugal ultrafiltration (Millipore membranes) having molecular weight cutoffs of 30, 10, and 3kDa. The fractions were defined according to molecular size of >30, 10-30, 3-10, and <3kDa. The percentage of molecular weight distribution of each peptide was expressed as the relative percentage of each yield.

Protein assay by Bicinchoninic Acid assay (BCA) method: The protein concentration of each fraction was determined by BCA assay [12]. BCA kit is contained reagent of A and B and Bovine Serum Albumin (BSA) as standard. This method especially is more applicable than other procedures for peptides and small protein fragments. To prepare the working reagent, mixed 50 parts BCA reagent A with 1 part of BCA reagent B (50:1, Reagent A:B) until it is light green in color. For the 96 well plate assay, 8 parts of the BCA Working reagent are mixed with 1 part of a protein sample. The sample is a blank (a BSA protein standard, or peptide fraction). After shaking the samples, the microplates were incubated at 37°C for 30min in dark. The absorbance at 562nm is recorded and the protein concentration is determined by comparison to a standard curve (200-1000mg/ml). Increasing protein concentrations produce proportionally deeper colors and higher absorbance.

Antiproliferative assay

Cell line and culture: 4T1 cell lines (Mouse breast tumor cells) were purchased from Pasteur Institute's Cell Bank and cultured at 37°C in RPMI 1640 (Gibco; USA), enriched with 10% FBS (Fetal Bovine Serum) and 1% penicillin and streptomycin (Ratio 1 to 1). Stocks were maintained in 25cm^2 tissue culture flasks to maintain in exponential phase. 10^4 cells were seeded in 96 well microplates and cultured for 24 hours at the incubator situation (CO_2, temperature).

Cytotoxicity and MTT assay: Cytotoxic activities were evaluated according to the method of Picot *et al*. [13] and were expressed as half Inhibitory Concentration (IC50), which defined as the tested sample concentration that inhibits 50% cell growth. Different concentration (0.2, 0.5, 0.8, 1.2mg mL^{-1}protein final concentration) of peptide fractions which was prepared in serum-free RPMI 1640 was added to the wells and Dimethyl Sulfoxide (DMSO; various concentrations of DMSO 10% mixed with RPMI) and untreated cells applied as a positive and negative control, respectively. The microplate was incubated in 5% CO_2-95% air for 24 and 48h. At the end of the incubation, the supernatant was removed from each well and 500µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma; USA) solution (5mg/mL) was added to each well and incubated for 3h. The MTT solution was then removed and 100µL of DMSO was added to dissolve the formazan crystals at room temperature for 30min. The optical density of wells was read at 570nm, using an ELISA reader (Biotech; USA). The effect of the samples on the proliferation of cells was expressed as the Cell Cytotoxicity (%). All experiments were performed at least twice in triplicate.

Cell viability (%)=[A(sample)/A(control)]×100

Cell Cytotoxicity (%)=100-Cell viability

Statistical analysis: All data were submitted to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test at p≤0.05. The data were analyzed using the SPSS 19.0.

Result and Discussion

Percentage of molecular weight distribution by ultrafiltration

Table 1 show the percentage of molecular weight distribution for the 4 fractions (>30kDa, 10-30 kDa, 3-10 kDa, and <3kDa). The highest value for <3kDa fraction was about 44.97% and the lowest one was recorded for 10-30kDa fraction with the value of 10%. During hydrolysis, a wide variety of smaller peptides and free amino acids are generated, depending on enzyme specificity. Hence, the high amounts peptides in the MW range of <10kDa in the current study (75%) demonstrated that the alcalase acted well in cleavage of peptide bonds and releasing the bioactive peptides from crab hydrolysate. Similarly, among the ultrafiltration fractions of loach (*Misgurnus anguillicaudatus*) protein hydrolysate (>10kDa, 5-10kDa, 3-5kDa and <3kDa), the fraction with a MW<3kDa

accounted for about 88% of the total hydrolysate [14]. In a similar case, the <3kDa fraction accounted for about 44% of the whole alcalase hydrolysate of sandfish (*Arctoscopus japonicus*) [15]. The authors indicated that these hydrolysates contained mostly low molecular weight peptides, coinciding with their high percent DH. Alcalase is proposed as effective endoproteinase for controlled hydrolysis due to the high degree of hydrolysis and also producing mainly small- and medium-sized oligopeptides or polypeptides with high bioactivity [16-18].

Table 1) Percentage of molecular weight distribution for the crab protein hydrolysate

Distribution (%)	Molecular weight
15.56±0.62^{d}	>30
10.00±0.65^{c}	10-30
29.43±0.83^{b}	3-10
44.97±0.92^{a}	<3

Values in the same column with different superscript letters are significantly different (p≤0.05).

Effect of time and peptide concentration on the cytotoxic activity

In this study, the cytotoxic activity of different MW peptide fractions from crab hydrolysate against the 4T1 cell line was investigated. The results showed that although all peptide fractions from the crab hydrolysate showed some activity, the viability of the cultured 4T1 cell was decreased in the presence of the peptide fractions 10-30 and >30kDa in an irregular manner. The inhibition ratio of >30kDa fraction showed more than 95% in all of the protein concentrations of this fraction. That could be because of the presence of unidentified compounds presented in these fractions may be toxic to cultured cells or affect metabolism and survival nonspecifically. Ultrafiltration is one of the most popular means of membrane filtration, which removes or concentrates the target components present in a solution. While the hydrolysate of crab was sequentially passed through UF membranes with the molecular weight cut-off of 30, 10, and 3kDa, a membrane with a smaller MW cut-off was better able to remove undissolved compounds and produce more pure peptide fractions respectively.

The 10-30kDa peptide fraction showed low cytotoxicity at the concentration of 1.2mg/ml. The reason for no cytotoxic effect of this peptide fraction in a dose-dependent manner could be attributed to the fact that the other concentration which used for this peptide fraction was not sufficient to show any effect. More ever, it could be assumed that there are no peptide sequences with cytotoxic effect against the 4T1 cell in 10-30kDa fraction.

Interestingly, the cell viability of cultured 4T1 was significantly reduced by the addition of the peptide fraction of <3 and 3-10kDa in dose-dependent and was significantly different in the 24 and 48h (Diagram 1 and 2). The IC50 values were about 0.40±0.063mg mL^{-1} for <3kDa and 0.25±0.026mg mL^{-1} for 3-10kDa peptide fractions at 48h (Table 2).

Table 2) Cytotoxic activity ($IC50^{a}$) of peptides against 4T1 cell line at 24 and 48h

Fraction	**IC50** (mg/ml) **24h**	**IC50** (mg/ml) **48h**
3-10	0.39±0.042^{a}	0.25±0.026^{A}
< 3	0.89±0.098^{b}	0.40±0.063^{B}

^{a}IC50 represents the concentration of peptides at which cell viability was inhibited by 50%.; The MTT assay was repeated in triplicate, and IC50 value was determined by averaging three repeated experiments.

Several studies revealed that anticancer agents have a special dose-response curve, which can show the most effective inhibition against tumor cells at a defined concentration which is non-lethal to normal tissue. The peptide fraction isolated from pepsin hydrolysate of algae protein waste had strong dose-dependent antiproliferation (IC50 1.74mg/mL) and induced a post-G1 cell cycle arrest in AGS cells [19]. Hexapeptide FIMGPY from skate cartilage protein hydrolysate showed strong, dose-dependent cytotoxicity against HeLa cell lines, with an IC_{50} of 4.81mg/mL [20]. A peptide fraction (3.6kDa) from solitary tunicate hydrolysate showed potent anticancer activity against AGS, DLD-1, and HeLa cancer cells (IC50 577.1, 163.3, and 887.2µg/mL) [21]. Although the IC_{50} of low molecular weight peptide fractions of rocky shore crab was lower than these studies, the value is more than that of commercial standard.

Effect of molecular weight on cytotoxic activity

There is still a shortage of solid evidence to clarify the relationship between the structural properties of peptides and their anticancer property. However, hydrophobicity, molecular

size, amino acid composition, and sequence are deemed to play an essential role in the bioactivity of peptides [20]. It was proposed that peptide fractions with molecular weights ranging from 400-1400 Da exhibit the strongest antiproliferative activity [22]. Our result confirmed that both low MW peptide fractions (3-10 and 3kDa) showed a remarkable cytotoxic effect against the against 4T1 cells.

The <3kDa peptide fraction of sea cucumber was the most potent antiproliferative fraction among other higher MW fractions at the highest concentration (5.5mgmL^{-1}) [23]. The MW of the best antiproliferative peptide obtained from algae protein waste was 1157 [19]. The low molecular weight peptides have greater molecular mobility and diffusivity than the high molecular weight peptides, which appears to improve interactions with cancer cell components and enhances anticancer activity [22]. However, the molecular weight cannot be considered the most important factor influencing anticancer activity, since our result indicated that although both low MW peptide fractions showed the maximum percentage of cytotoxicity with the value of (70±1.17%) for 3-10kDa fraction and (64.3±1.2%) for <3kDa fraction at 1.2mg mL^{-1} (48h), with no statistically significant differences with each other (Diagram 1 and 2), but the 3-10 kDa fraction was more potent than <3kDa fraction (p≤0.05) with lower IC_{50} value in 24 and 48 h (Table 2). As shown in Diagram 3, DMSO as a positive control with toxic effect against cells, showed the same cytotoxicity at the concentration of 25% (72±1.4%).

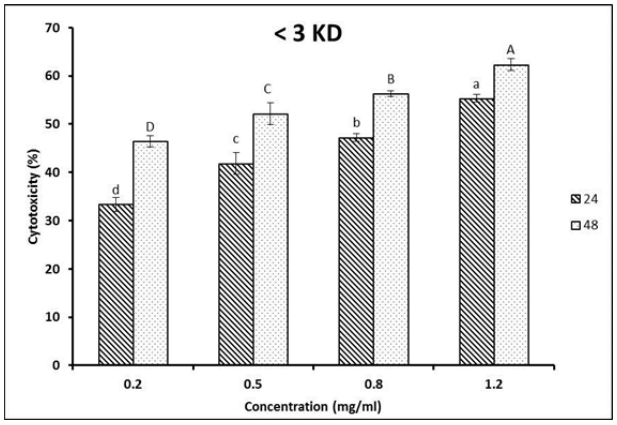

Diagram 1) Cytotoxicity of 4T1 cells after treating with various concentration of <3kDa fraction. Bars (mean±standard deviation, n=3) with different letters have mean values that are significantly different at P≤0.05.

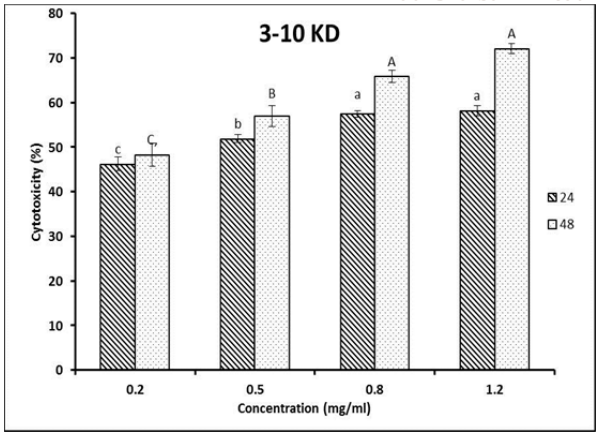

Diagram 2) Cytotoxicity of 4T1 cells after treating with various concentration of 3-10kDa fraction; Bars (mean±standard deviation, n=3) with different letters have mean values that are significantly different at p≤0.05

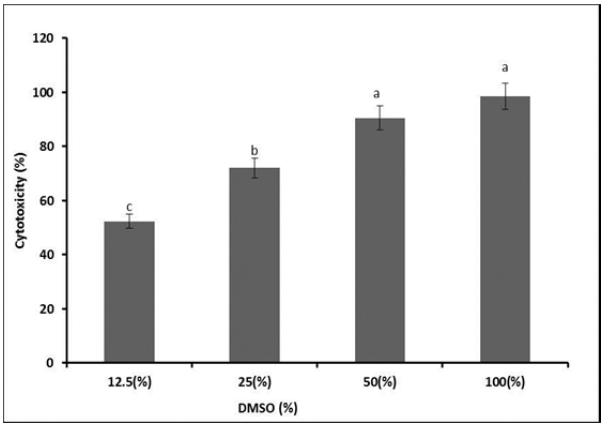

Diagram 3) Cytotoxicity of 4T1 cells after treating with various concentration of DMSO; All data are presented as the mean±SD of triplicate results

According to several studies, besides peptide size, the anticancer nature of the peptides is highly dependent on amino acid composition. It was proposed that the hydrophobic properties of the protein hydrolysates and/or peptides could play important roles in their anticancer activities. Song et al. [24] reported that hydrophobic residues of Alanin and Leucin in the peptide YALPAH were considered one of the important factors for the peptide's antiproliferative activity. Another study showed that BCP-A peptide which was purified from <1kDa fraction of protein hydrolysate of clam (*Tegillarca granosa*) exhibited cytotoxic activity against different cancer cells (PC-3, HeLa, DU-145 and H-1299 cells) based on its hydrophobicity [6]. The hydrophobic amino acids with positive charge could lead to increased interaction between peptides and the outer leaflets of tumor cell membrane bilayers

(high phospholipid contents) with negative charge lead to cytotoxicity effect against tumor cells [6, 20]. Alcalase has been proved as an effective protease for producing bioactive peptides due to the specificity towards hydrophobic amino acids and small-sized peptides [25-28].

Therefore, according to our results, the cytotoxic activities of peptide fractions obtained from the rocky shore crab (*Grapsus albacarinous*) could be varied through molecular weights and amino acid contents. Even though the exact mechanism by which low molecular weight peptide fractions of 3-10 kDa and < 3 kDa, act as antiproliferative was not clearly known, but according to the alcalase specificity towards small-sized peptides with hydrophobic residue, we could assume that the alcalase hydrolysis of crab protein generating the small-sized peptides fractions with hydrophobic amino acid contents lead to cytotoxic effect against 4T1 cancer cells.

The limitations of this research include the difficulty in determining the amino acid sequence of anticancer peptides from rocky shore crab *Grapsus albolineatus* and we suggested further purification and characterization of low molecular weight peptide fractions from rocky shore crab *Grapsus albolineatus* for identification of novel anticancer peptides.

Conclusion

In this study, the cytotoxic activities of the different molecular weight peptide fractions from rocky shore crab *Grapsus albolineatus* protein hydrolysate were evaluated on 4T1 cells. The result demonstrated that the low molecular weight peptide fractions (3-10 kDa and < 3 kDa) from alcalase hydrolysis of the crab proteins showed more potent cytotoxicity than that of > 30 kDa and 10-30 kDa fractions. These results suggested that the low molecular weight peptide fractions have a potent high anticancer activity that could be beneficially used as a natural source of healthy compounds. Further research should be done in order to purify and identify anticancer peptides from rocky shore crab *Grapsus albolineatus* protein hydrolysate.

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References

1- 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):963-86.

2- Ding GF, Huang FF, Yang ZS, Yu D, Yang YF. Anticancer activity of an oligopeptide isolated from hydrolysates of Sepia ink. Chin J of Nat Medicines. 2011;9(2):151-5.

3- Bazinet L, Doyen A. Antioxidants, mechanisms, and recovery by membrane processes. Crit Rev Food Sci Nutr. 2017;57(4):677-700.

4- Mehaia MA, Cheryan M. Membrane bioreactors: Enzyme processes. Biotechnol Food Process Eng. 1990;23:67-136.

5- Zheng LH, Wang YJ, Sheng J, Wang F, Zheng Y, Lin XK, Sun M. Antitumor peptides from marine organisms. Mar drugs. 2011;9(10):1840-59.

6- Chi CF, Hu FY, Wang B, Li T, Ding GF. Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (Tegillarca granosa) muscle. J Funct Foods. 2015;15:301-13.

7- Bishayee K, Khuda-Bukhsh AR, Huh SO. PLGA-loaded gold-nanoparticles precipitated with quercetin downregulate HDAC-Akt activities controlling proliferation and activate p53-ROS crosstalk to induce apoptosis in hepatocarcinoma cells. Mol Cells. 2015;38(6):518-27.

8- Maki A, Diwakaran H, Redman B, Al-Asfar S, Pettit GR, Mohammad RM, et al. The bcl-2 and p53 oncoproteins can be modulated by bryostatin 1 and dolastatins in human diffuse large cell lymphoma. Anticancer Drugs. 1995;6(3):392-7.

9- Huang F, Yang Z, Yu D, Wang J, Li R, Ding G. Sepia ink oligopeptide induces apoptosis in prostate cancer cell lines via caspase-3 activation and elevation of Bax/Bcl-2 ratio. Mar Drugs. 2012;10(10):2153-65.

10- Han Y, Cui Z, Li YH, Hsu WH, Lee BH. In vitro and in vivo anticancer activity of pardaxin against proliferation and growth of oral squamous cell carcinoma. Mar Drugs. 2016;14(1):2.

11- Pino-Angeles A, Leveritt III JM, Lazaridis T. Pore structure and synergy in antimicrobial peptides of the magainin family. PLoS Comput Biol. 2016;12(1):e1004570.

12- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano M, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985;150(1):76-85.
13- Picot L, Bordenave S, Didelot S, Fruitier-Arnaudin I, Sannier F, Thorkelsson G, et al. Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. Process Biochem. 2006;41(5):1217-22.
14- You L, Zhao M, Regenstein JM, Ren J. Purification and identification of antioxidative peptides from loach (Misgurnus anguillicaudatus) protein hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. Food Res Int. 2010;43(4):1167-73.
15- Jang HL, Liceaga AM, Yoon KY. Purification, characterisation and stability of an antioxidant peptide derived from sandfish (Arctoscopus japonicus) protein hydrolysates. J Funct Foods. 2016;20:433-42.
16- Kristinsson HG, Rasco BA. Fish protein hydrolysates: production, biochemical, and functional properties. Crit Rev Food Sci Nutr. 2000;40(1):43-81.
17- You L, Zhao M, Cui C, Zhao H, Yang B. Effect of degree of hydrolysis on the antioxidant activity of loach (Misgurnus anguillicaudatus) protein hydrolysates. Innov Food Sci Emerg Technol. 2009;10(2):235-40.
18- Zhang L, Liu Y, Tian X, Tian Z. Antimicrobial capacity and antioxidant activity of enzymatic hydrolysates of protein from rushan bay oyster (Crassostrea gigas). J Food Process Presrv. 2015;39(4):404-12.
19- 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):1202-7.
20- Pan X, Zhao YQ, Hu FY, Chi CF, Wang B. Anticancer activity of a hexapeptide from skate (Raja porosa) cartilage protein hydrolysate in HeLa Cells. Mar Drugs. 2016;14(8):E153.
21- Jumeri, Kim SM. Antioxidant and anticancer activities of enzymatic hydrolysates of solitary tunicate (Styela clava). Food Sci Biotechnol. 2011;20(4):1075.
22- Wang L, Dong C, Li X, Han W, Su X. Anticancer potential of bioactive peptides from animal sources. Oncol Rep. 2017;38(2):637-51.
23- Pérez-Vega JA, Olivera-Castillo L, Gómez-Ruiz JÁ, Hernández-Ledesma B. Release of multifunctional peptides by gastrointestinal digestion of sea cucumber (Isostichopus badionotus). J Funct Foods. 2013;5(2):869-77.
24- Song R, Wei RB, Luo HY, Yang ZS. Isolation and identification of an antiproliferative peptide derived from heated products of peptic hydrolysates of half-fin anchovy (Setipinna taty). J Funct Foods. 2014;10:104-11.
25- Gallegos-Tintoré S, Torres-Fuentes C, Martínez-Ayala AL, Solorza-Feria J, Alaiz M, Girón-Calle J. Antioxidant and chelating activity of Jatropha curcas L. protein hydrolysates. J Sci Food Agric. 2011;91(9):1618-24.
26- Kasper JR, Andrews EC, Park C. Product inhibition in native-state proteolysis. PLoS one. 2014;9(10):e111416.
27- Song L, Li T, Yu R, Yan C, Ren S, Zhao Y. Antioxidant activities of hydrolysates of Arca subcrenata prepared with three proteases. Mar Drugs. 2008;6(4):607-19.
28- Ramezanzade L, Hosseini SF, Nikkhah M, Arab-Tehrany E. Recovery of Bioactive Peptide Fractions from Rainbow Trout (Oncorhynchus mykiss) Processing Waste Hydrolysate. Ecopersia. 2018;15:6(1):31-40.