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Research Article

Bioactive peptides from the Pacific white-leg shrimp (*Litopenaeus vannamei*) induce apoptosis and anticancer activities in HCT-116 colon cancer cell line

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Abstract

In this study, the effects of white-leg shrimp hydrolysate protein extracted in a progressive rise in temperature (40-60°C) (Gradual) and without centrifugation (Mix) on the colon cancer cells were determined. Both bioactive peptides were prepared using enzymatic hydrolysis with alcalase. The cytotoxic effect on HCT-116 cell line was evaluated using the Neutral red and MTT assay. *In-vitro*, antioxidant activity was performed using DPPH, TAC, and nitric oxide assays. Apoptosis with acridine orange/etho bromide and redox changes was evaluated in the cell lines.The results of toxicity assays showed that the survival rate of the cells were decreased by increasing the concentrations of the peptides (0.3, 0.6, 1.2, and 2.4 mg/mL). Based on the results of antioxidant activity (TAC, DPPH, No), the Gradual peptides had significantly higher antioxidant activity than Mix peptides (p<0.05). In addition, the Gradual peptide increased the concentration of nitric oxide compared to the control and Mix groups (p<0.05). Results also showed that the Gradual peptides reduced the secretion of catalase and GSH enzymes in the cancer cells (p<0.05). Both types of peptides increased apoptosis in the cancerous cell line and a higher value was observed in bioactive peptides treatments The results of the present study showed that the Pacific white shrimp hydrolysates protein obtained in a progressive rise in temperature showed anti-cancer activities against colon HCT-116cancer cell line.

Keywords: Peptides, Processing by-products; Colon cancer, Anticancer activity, *Litopenaeus vannamei*

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Introduction

It is estimated that 50,000 new cases of cancer are diagnosed annually in Iran, and a significant number of cases are related to gastrointestinal cancers (Mohebbi *et al*., 2008). This is the third and fourth most common cancer in Iranian males and females, respectively (Alireza *et al*., 2005). It is also the second leading cause of death in the Western countries (Sodeifi *et al*., 2006). Today, aggressive procedures such as chemotherapy and radiation therapy are used to treat and kill cancer cells. These procedures have many complications such as pain, hair loss, damages to the body's normal cells, and disruptions of various physiological functions (Mousavi *et al*., 2009). The side effects of chemical medicines have led researchers via the world to develop the techniques based on natural antioxidants and functional foods as adjunctive to primary treatments.

Seafood protein hydrolysates containing low-molecular-weight peptides showed various biological functions such as antioxidant, anti-thrombotic, antihypertensive, anti-cancer, and immunomodulatory effects (Nikoo *et al*., 2014; Sun *et al*., 2020; Sarteshnizi *et al*., 2021). Therefore, they may be used as a promising ingredient for developing functional foods due to their multiple health benefits in the host (Sun *et al*., 2020). Thus, the potential nutritional and biological significance of fish proteins and peptides have driven the interest in the production of "functional" foods (Le Gouic *et al*., 2018). They are extracted from different food proteins including fish, milk, soybeans, egg, meat, and seaweeds. Bioactive ppetides contain 2 to 20 amino acids with molecular mass less than 3 kDa and their antioxidant activity depends on their amino acid sequences and peptide size (Aluko, 2015; Nikoo and Benjakul, 2015).

Antioxidant activity and inhibitory growth effects of bioactive peptides on liver, lungs, and colon cancer cells have been reported in several studies (Koehler *et al*., 2011). Those studies have demonstrated that food-drived peptides could inhibit cancer cells' growth due to their strong antioxidant properties (Rayaprolu *et al*., 2013).

Pacific white shrimp (*Litopenaeus vannamei*) is an important aquaculture species around the world. 45-48% of shrimp weight is by-products, which are a potentially rich source of functional ingredients. Among the functional products, bioactive peptides fractions showed high nutritional value and antioxidant activity (Sila *et al*., 2014; Ambigaipalan and Shahidi, 2017). The aim of this study was to investigate the effects of Pacific white shrimp bioactive peptides on apoptosis and stress oxidative on the colon cancer cell line HCT-116.

Materials and methods

Preparation of protein hydrolysates

Farm-raised Pacific white shrimp (18±2 g) were purchased from a shrimp processing plant. The shrimp by-products (500 g) were minced with a meat grinder using a 3 mm hole plate (Pars Khazar Co., Tehran, Iran) and

then, mixed at a 1:1 ratio with a distilled water (500 mL) and homogenized for 2 min with a Heidolph DIAX 900 homogenizer (Heidolph Instruments GmbH, Schwabach, Germany) at speed 4. Hydrolysis was done according to the method of Nikoo *et al*. (2021) at an initial pH (~7.1) using gradualy increasing temperature (Gradual) (40-60 ºC) for 3 h (9 ºC/min). During the autolysis, the mixture was continuously stirred using an overhead stirrer. Reactions was stoped by boiling water bath (~95 ºC) for 10 min in the mixtures, filtered using two layers of cheesecloth followed by centrifugation at 4000× *g* for 20 min at 4 ºC. The supernatants were freeze-dried and the part of the hydrolysates solution that were dried without centrifugation were refereed to as "Mix" peptide sample.

Cell culture

The HCT116 cell line (Pasteur Institute, Iran) was used in this study. These cells were cultured under special conditions (37°C, 10% bovine fetal serum, streptomycin 100 µg/mL, and 5% CO2 pressure) in RPMI 1640 medium. Effective drug concentrations (concentrations close to IC50) were used to improve the evaluation of bioactive peptide effects. Untreated cells were used in the control group. It should be noted that the tests were repeated three times to improve the statistical certainty.

Determination of cytotoxicity

MTT and Neutral Red assay were used to determine the optimal drug concentration and cell viability (IC50). A peptide concentration of 25 mg was dissolved in 1 mL of saline phosphate buffer and sterilized with a 0.2-micron filter to conduct the MTT assay. The concentrations of 0.3, 0.6, 1.2 and 2.4 mg/mL were used in 96 microplate wells containing 5000 HCT116 cell line. 10 µL of MTT assay solution was added to the cultures, four hours before cell extraction,. Since these solutions dissolve the purple-colored formazan deposition in normal cells, the absorption rate was measured by ELISA Plate reader at 570 nm wavelength. Similar concentrations were used in the culture medium for the Neutral red assay. The 1x neutral red stains were added to the culture medium in a 1:10 ratio and the cells were incubated for 4 h at 37°C. A solution of glacial acetic acid - ethanol (96% alcohol 50%, acetic acid 4%, and distilled water 49%) were added to the cells culture and the light absorption rate created at 540 nm wavelength was read by Elisa Reader after several sampling (Repetto *et al*., 2008).

The cell line (10^7) were washed with PBS solution and lysed using cellular lysate buffer (100 µL). The lysed cells were placed on ice for 30 min and vortexed for 15 s, every 10 min. Finally, the lysed solution was centrifuged for 5 min at 13000 rpm and the supernatants were used for subsequent measurements.

In-vitro Antioxidant activity of bioactive peptides

Based on the results of MTT assay and neutral red tests, 1.2 mg/mL concentrate of peptides was used to evaluate their *In-*

vitro antioxidant activities. The antioxidant characteristics of peptides were evaluated using special kits from Arsam Fara Zist Company (Arsam fara zist, Urmia, Iran). The DPPH Scavenging Capacity, NO (nitric oxid), and TAC (total antioxidant activity) tests were evaluated according to manufacturer instructions. To assess the DPPH scavenging capacity of antioxidants, 50 µL of different concentrates were added to 450 µL of phosphate buffer solution and 500 µL of DPPH solution were added as the source of free radicals. The samples were then incubated in the dark for 30 min at ambient temperature, and finally, the adsorption rate was read at 517 nm wavelength. Purple to yellow conversion along with the decrease in adsorption at 517 nm has a direct correlation to the protonation power of antioxidants. TAC was measured using the ABTS method. In this method, ABTS is oxidized to green $ABTS^+$ in the presence of a suitable oxidant, and the process is inhibited by the presence of antioxidants. The total antioxidant capacity (TAC) of the sample can be determined by measuring $ABTS^+$ absorption at 414 nm. The final product of NO, NO_2^-, was used to measure NO. The NO assay kit works based on the following reaction where NO_2^- reacts with sulfanilamide and 1-N-naphthyl ethylenediamine dichloride NED under acidic conditions (phosphoric acid) to produce the azo compound.

Changes in redox status of HCT116 cancerous cell line

After measuring the *in-vitro* antioxidant activity of the bioactive peptides, redox changes of the HCT116 cell line were also evaluated along with bioactive peptides. Then itric oxide (NO), glutathione reduction (GSH), catalase (CAT), and lipid peroxidation (MDA) tests were used for this purpose. The instructions of the Arsam Farazist kit were followed for the nitric oxide test. In the glutathione reduction (GSH) test, the thiol in glutathione reacts with the component reagent, dithio-base nitrobenzoic acid (DTNB) to produce yellowish nitrobenzoate (TNB), which can be quantified at 412 nm. Color intensity is directly proportional to the reduction of thiols in the sample. The peroxidation function of catalase was used to measure catalase. The formaldehyde produced in this reaction was measured by the colorimetric method. The lipid peroxidation is one of the cellular damage mechanisms in animals and plants, and this kind of damage is measured based on the concentration of Malondialdehyde. The TBARS (thiobarbituric acid reactive substance) test is a direct quantitative method for measuring the MDA from biological samples. Specimens with MDA and standard samples first react with TBA at 95°C. After incubation for a few minutes, the specimens and standards can be measured with a spectrophotometer or fluorimeter. The MDA value of unknown samples can be determined by comparing it to the standard MDA curve.

Acridine orange/ethidium bromide staining for morphological observation of apoptosis

Fifty thousand cells were cultured in 12-segment pellets, and the cells were treated with peptides after 24 h. Then, the cells were collected and cell pellets were dissolved in a saline phosphate buffer for Acridine Orange/ Ethidium Bromide staining. In this process, 100 µg of both ethidium bromide and acridine orange stains were dissolved separately in 1 mL PBS. Then, a volume ratio of 1:1 of two solutions was prepared and used during the experiment. The cellular suspension was mixed with 1 µL of acridine orange-ethidium bromide solution, the cells were coated with a slide and evaluated under a fluorescence microscope. At least 400 to 600 cells were counted to determine the percentage of apoptotic cells.

Statistical analysis

All experiments were conducted in three replications and the data analysis was performed using Spss V.22. One way-ANOVA test was used to compare the average values and the Duncan discriminant test was implemented to separate statistically significant means. The data were also displayed as mean and standard deviations.

Results

Cytotoxicity based on vital neutral red and MTT assay

Figure 1 shows the toxicity of Mix and Gradual bioactive peptides based on MTT and Neutral red tests in the HCT116 cell line. Four concentrations of the peptides (0.3, 0.6, 1.2, and 2.4mg/mL) were used in cell culture mediu4m to evaluate their toxicity. The results demonstrated that the viability of cells decreases with increasing the concentration of peptides. Based on these results, both Mix and Gradual peptides in 1.2 mg/mL had a lethality close to 50%, thus, this concentration was selected for further studies on both types of peptides.

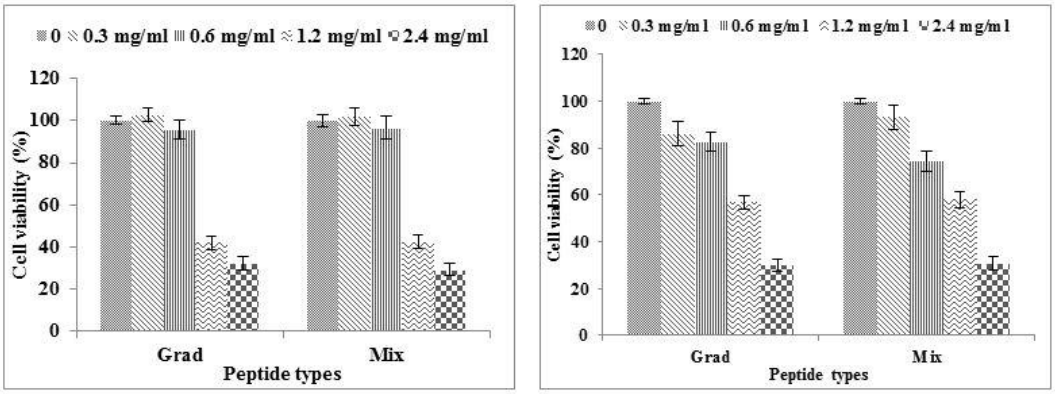

Figure 1: The results of cytotoxicity of Mix and Gradual bioactive peptides based on MTT staining (right) and Neutral red (left) used in this study at selected concentrations.

Results for Mix and Gradual bioactive peptides In vitro antioxidant activity

Figure 2 shows the results for *In vitro* antioxidant activity of Gradual and Mix

at a concentration of 1.2 mg/mL. Based on the results, the Mix bioactive peptides had a low scavenging ability for DPPH radicals that were significantly different from Gradual ($p<0.05$). In assessing the Total antioxidant capacity (TAC), Mix bioactive peptides had a higher ability compared to Gradual peptides in scavenging oxygen and nitrogen free radicals and there was a significant difference in this regard for the two peptides ($p<0.05$). Similar results were obtained in the nitric oxide test and more activity was represented by Mix bioactive peptides compared to Gradual treatment and the difference was significant ($p<0.05$).

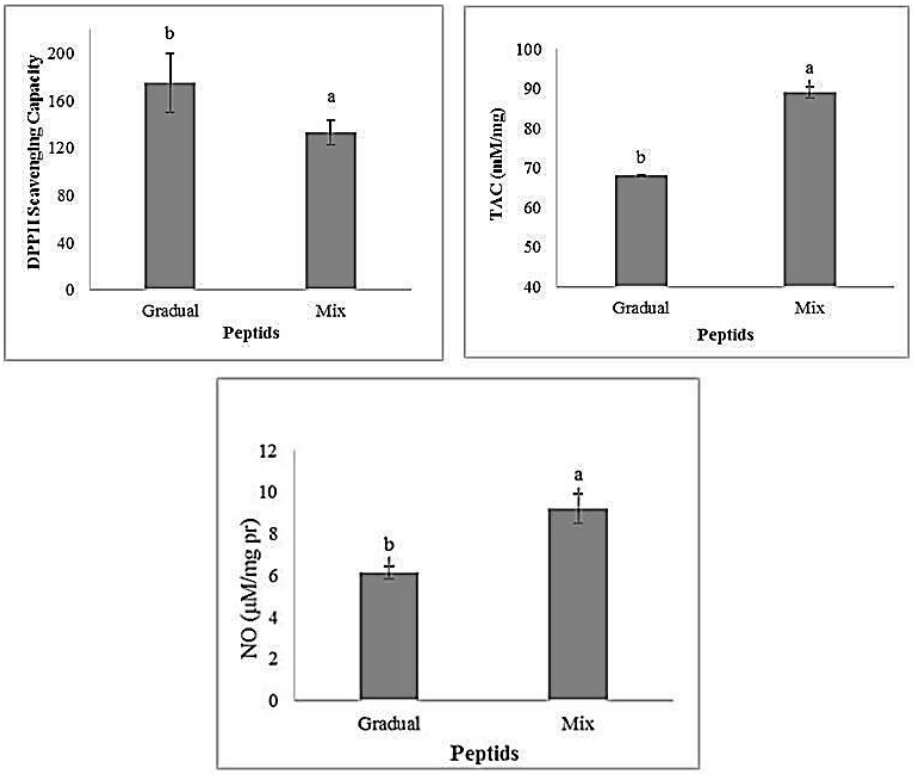

Figure 2: The results for *In vitro* antioxidant activity of Mix and Gradual bioactive peptides.

Redox status changes of HCT116 cancer cell line in the presence of Mix and Gradual bioactive peptides

Figure 3 shows the results for redox changes in HCT116 cancer cell lines exposed to the bioactive peptides used in this research. Measuring catalase activity and glutathione reduction rate showed that the application of bioactive peptides caused a significant reduction in the activity of these enzymes in cancerous cells ($p<0.05$). The results also showed that there is a significant difference between the Gradual and Mix bioactive peptides treatments and Gradual peptides have significantly lower GSH and catalase than Mix peptides ($p<0.05$). The results for using bioactive peptides demonstrated a significant effect on the amount of nitric oxide in treated samples compared to the control group ($p<0.05$). The results also showed that using Gradual and Mix bioactive peptides reduces the amount of

nitric oxide significantly compared to the control group ($p<0.05$). There was a significant difference between Gradual and Mix bioactive peptides treatments and the peptides processed in Gradual temperature had significantly higher NO than the Mix sample ($p<0.05$). Malondialdehyde (MDA) concentration test results showed that using Mix peptide significantly increases the concentration of this substance compared to Gradual peptide and control treatments ($p<0.05$). No significant difference was observed between Gradual bioactive peptides and control treatments ($p>0.05$).

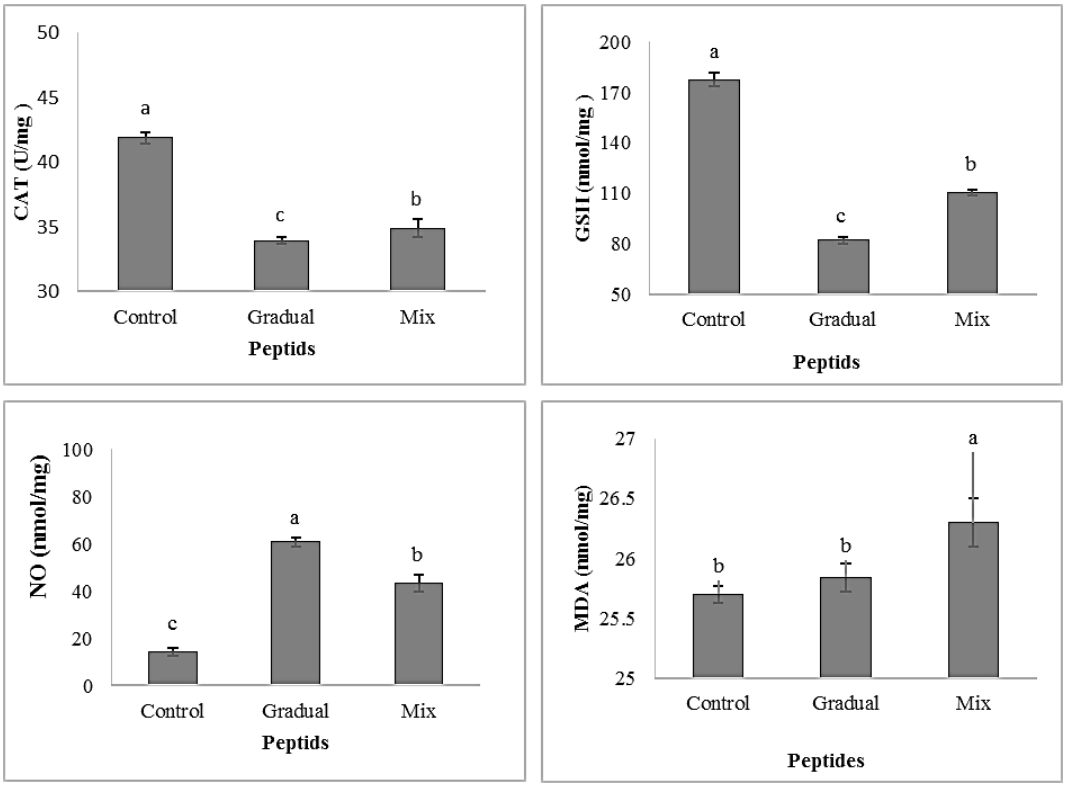

Figure 3: The Results for reduced glutathione, nitric oxide, malondialdehyde concentration, and catalase activity of HCT116 cancerous cells exposed to Mix and Gradual bioactive peptides.

Evaluation of morphological changes in HCT116 cell line treated with gradual and mixed peptides

Cellular death was quite evident in peptide-treated cells and reflected in changes in shape, size, and detachment of cells from the surface. Quantitative analysis of apoptosis by acridine orange/etho bromide (AO/EtBr) was conducted using a fluorescence microscopic study. UniformLy bright green nuclei represented living cells. However, the bright green and orange area of dense or fragmented chromatin in nuclei of apoptotic cells and uniformLy bright orange nuclei showed necrotic cells. The apoptosis rate of cells treated with Gradual and Mix peptides compared to control cells showed that peptide treatment with Gradual and Mix

increased the apoptosis rate by 75.5% and 76%, respectively (Table 1 and Fig. 4).

Table 1: The evaluation of cell death mechanisms induced by Mix and Gradual peptides in HCT116 cell line based on acridine orange/ethidium bromide staining (n=3).

Groups	Normal (%)	Necrosis (%)	Apoptosis (%)
Control	96.03	0.98	2.91
Mix	8.32	10	76
Gradual	66.37	7.5	75.5

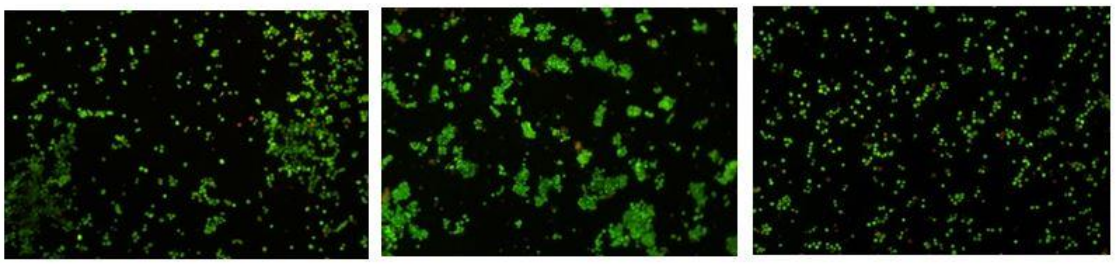

Figure 4: The evaluation of apoptosis mechanism produced by control (left), Mix (middle), and Gradual peptides (right) in HCT116 cells using Acridine Orange-Ethidium Bromide staining.

Discussion

The results of this study indicated that Gradual and Mix bioactive peptides have optimal antioxidant effects. Moreover, Gradual bioactive peptides have a high scavenging ability for DPPH radicals as well as nitric oxide from their environment. They were also significantly different with Mix bioactive peptides extracted. Conversely, Mix bioactive peptides had higher antioxidant capacity, which contradicts the recent results. Some studies show that based on the methodology, there are different results for the antioxidant capacity of peptides (Rabiei *et al*., 2019). The antioxidant capacity of the same peptide can be different in FRAP or DPPH methods. Several studies have also demonstrated that bioactive peptides exracted from Mackerel fish viscera (Kumar and Maranas, 2009), Hick fish fillet (Chen *et al*., 1996), Crocker fish muscle (Chi *et al*., 2015), myofibrillar protein of Patina fish (Najafian and Babji, 2014) show antioxidant and anti-redox effects. The results of the present study are consistent with the aformentioned research findings.

Our findings also showed that Gradual peptides increased the nitric oxide ions (NO^-), subsequently, the catalase and glutathione reduction enzymes were significantly reduced in the treated cancerous cells. It can make these cells vulnerable to the body's immune and adaptability factors as the immune system and the cellular natural defense mechanisms destroy these cancerous cells with lower energy demand. According to Nikoo *et al*. (2021) analyzing Gradual peptide, the peptides with lower than 1000 daltons

molecular weight were predominant and dipeptides and tripeptides characteristics with a molecular weight of 180 to 500 daltons were the most abundant peptide fractions in this sample. Such a high fraction of dipeptides and tripeptides along with 30% of free amino acids in Gradual peptide has a high potential for free radicals scavenging in the above treatment, which contradicts the results of the present study. According to Aleman *et al*. (2011) findings, peptides with lower molecular weight have higher chelating activity than peptides with higher molecular weights. In contrast, Bamdad *et al*. (2011) reported that peptides with higher molecular weight increase the antioxidant activity of their environment by trapping free radicals in their peptide chains. The discrepancies in the results related to bioactive peptides indicate that molecular weight cannot be a determining factor regarding the antioxidant activity of peptides. Moreover, other amino acid factors may affect their oxidative or anti-oxidative activity including their type, sequence in the peptide chain, and special placement of specific amino acids in C and N positions (Harnedy and FitzGerald, 2012). Findings of Shaughnessy *et al*. (2006), also show that bioactive peptides can acidify the cytoplasm of cancerous cells of the colon by interfering with lysosome function and development of large vacuoles and induce ancholytic process instead of programmed cell death.

In the present study, MTT and Neutral Red methods were used to evaluate the cytotoxicity of peptides. MTT method is principally based on mitochondrial activity. Mitochondrial activity is stable in living cells. Consequently, increases or decreases in the number of living cells are linearly related to mitochondrial. The principles of the Neutral Red method are also based on the absorption of Neutral Red stain by normal cells. Among the studied concentrations for MTT and Neutral Red methods, the best concentration found for both peptides (Mix and Gradual) was 1.2 mg/µL and these two peptides had a lethality close to 50% in this concentration. Thus, 1.2 mg/µL was used as the selected concentration for further experiments. The present study findings on the lethal concentration for cancerous are consistent with the results of studies performed by Nuchprapha *et al*. (2020). The results of this project showed that the peptides processed and extracted from Pacific white shrimp under increasing temperature (40 to 60°C), can be used as oxidative agent in the treatment of colon cancerous cell line HCT-116 and further studies to investigate exaxt effective mechanism are needed.

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