Antioxidant and cytotoxic properties of protein hydrolysates obtained from enzymatic hydrolysis of Klunzinger's mullet (*Liza klunzingeri*) muscle

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Today, consumers are looking for functional foods that promote health and prevent certain diseases in addition to provide nutritional requirements. This study aimed to evaluate the antioxidant and cytotoxic properties of *Liza klunzingeri* protein hydrolysates. Fish protein hydrolysates (FPHs) were prepared from *L. klunzingeri* muscle using enzymatic hydrolysis with papain at enzyme/substrate ratios of 1:25 and 1:50 for 45, 90 and 180 min. The antioxidant activities of the FPHs were investigated through five antioxidant assays. The cytotoxic effects on $4T_1$ carcinoma cell line were also evaluated. The amino acid composition and molecular weight distribution of the hydrolysate with the highest antioxidant activity were determined by HPLC. All six FPHs exhibited good scavenging activity on ABTS (IC_{50}=0.60-0.12 mg/mL), DPPH (IC_{50}= 3.18-2.08 mg/mL), and hydroxyl (IC_{50}=4.13-2.07 mg/mL) radicals. They also showed moderate Fe^{+2} chelating capacity (IC_{50}=2.12-12.60 mg/mL) and relatively poor ferric reducing activity (absorbance at 70 nm= 0.01-0.15, 5 mg/mL). In addition, all hydrolysates showed cytotoxic activities against the $4T_1$ cells (IC_{50}=1.62-2.61 mg/mL). 94.6% of peptide in hydrolysate with the highest antioxidant activity had molecular weight less than 1,000 Da. *L. klunzingeri* protein hydrolysates show significant antioxidant and anticancer activities *in vitro* and are suggested to be used in animal studies.

Keywords: Antioxidant activity. Cytotoxic effect. Protein hydrolysate. *Liza klunzingeri.*

INTRODUCTION

In biological systems, free radicals are typically derived from the oxygen, nitrogen, and sulfur molecules. Due to their unpaired electrons, free radicals exhibit a great deal of combination desire for reaction with other molecules. The most important free radical including Reactive Oxygen and Nitrogen Species (ROS/RNS) are naturally produced by various metabolic pathways such as the aerobic metabolism in mitochondrial respiratory chain, and play numerous physiological roles such as intracellular signaling, regulation of cell proliferation and apoptosis, induction of gene expression, and ion transferring (Sarmadi, Ismail, 2010).

However, excessive production of these compounds under certain conditions can exert harmful effects by causing oxidative damage to important cellular structures. The ROS and RNS radicals react with nucleic acids, the side chains of amino acid in proteins, and double bonds of unsaturated fatty acids, triggering and developing oxidative stress, which plays an important role in the pathogenesis of many human diseases, including cancer (Nikoo, Benjakul, 2015). ROS contribute to tumor development and progression through two possible pathways inducing mutation of key gene and/or alterations of signaling and transcriptional pathways. When the cell with oxidized or otherwise modified DNA is divided, its metabolism and proliferation are impaired and a mutation occurs which is an important factor for carcinogenesis. In addition, products of lipid peroxidation can react with

metal ions and produce active compounds, such as epoxide and aldehyde, which cause mutations in the DNA of cells (Noda, Wakasugi, 2001).

Today, researchers have proven that daily diets play an important role in preventing, developing, and treating various types of cancers. The consumption of foods rich in natural antioxidants, such as vitamins E and C, can prevent the development of certain cancers by inhibition of free radicals and ROS (Terry *et al.*, 2000; Venugopal, 2008). Increased concerns about the association between health and diet have led to growing consumer demand for the health-promoting and functional foods. Functional foods are defined as food products that provide health benefits in addition to meeting basic nutritional needs of the body (Shahidi, Alasalvar, 2011). The global functional foods market size was 299 billion dollars in 2017 and is expected to reach 441 billion dollars in 2022 (Menrad, 2003).

Marine animals, that comprise about half of the world's biodiversity, provide a valuable source of bioactive and functional compounds. Some of these compounds have a proteinaceous nature and includes proteins, peptides, and amino acids. Marine animals, in addition to being an important source of high-quality protein, are also used as the raw material for production of physiologically important peptides (Raghavan, Kristinsson, Leeuwenburgh, 2008). Bioactive peptides are specific protein fragments which remain inactive within the sequence of their parent protein until released by enzymatic hydrolysis (Harnedy, FitzGerald, 2012). Bioactive peptides derived from marine animal using enzymatic hydrolysis exhibit numerous physiological functions such as immunomodulatory, antimicrobial, anxiolytic, and hypotensive activity (Giri, Ohshima, 2012; Kumar, Nazeer, Jaiganesh, 2011).

According to the FAO, the total catch amount of Mugilidae species from southern and southwestern waters of Iran was 9300 tons in 2017 and Klunzinger's mullet *(Liza klunzingeri)* capture comprise about 2950 tons of this amount (FAO, 2016). Klunzinger's mullet is an inexpensive and low-value fish due to its small size and the presence of a dark brown to black peritoneum (Kiabi, Abdoli, Naderi, 1999). The use of Klunzinger's mullet for the production of protein hydrolysates provide added value and allows the optimal use of marine resources that are decreasing. In this study, *L. klunzingeri* muscle protein was hydrolysed by papain and the antioxidant and cytotoxic effects of protein hydrolysates were studied *in vitro*. In addition, the molecular weight and amino acid sequence of the hydrolysate with the highest antioxidant activity was determined using HPLC.

MATERIAL AND METHODS

Material

2,2'-azinobios-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4′′-disulfonic acid sodium salt), Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Butylated hydroxytoluene (BHT), and Trypsin-EDTA solution were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other chemicals were purchased from Merck Co. (Germany) and were of analytical grade. Papain from papaya latex (1.5-10 units/mg Solid, pH 6.0, 40 °C) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The enzyme was stored at 4 °C until use.

Methods

L. klunzingeri proximate chemical composition: Fresh *L. klunzingeri* was provided from the fish market and transferred to the laboratory in ice. The fish were first washed and filleted, and the fillets were then minced and stored at -20 °C until the experiments. In order to determine the moisture content, 2 g of fish mince were dried in an oven at 105 °C to reach constant weight, the moisture content was calculated by measuring the weight loss following heating. Ash content was determined by complete oxidation of organic matter at 550-600 °C in a furnace. The nitrogen of the samples was determined via the Kjeldahl method. Crude protein was calculated by multiplying the determined nitrogen content by a nitrogen-to-protein conversion factor ($\times$ 6.25). Fat content of sample was determined by AOAC Soxhlet procedures (AOAC, 1995).

Preparation of protein hydrolysates

Samples of *L. klunzingeri* mince (50 g) was placed in an Erlenmeyer flask and then 100 mL of phosphate buffer (pH 6) was added to keep the pH constant throughout the incubation time. In order to inactivate the endogenous enzymes, the samples were heated in a water bath at 85 °C for 20 min. After cooling, samples were hydrolyzed using papain (enzyme to substrate ratio of 1:50 and 1:25) for 45, 90 and 180 at 55°C. The hydrolysis was performed using 250 mL glass vessels inside a shaking water bath (SWB 15 Precision). After the incubation time, samples were heated at 95 °C during 15 min to stop the enzymatic reaction. After cooling at room temperature for 15 min, the

samples were centrifuged (8000 ×g at 10 °C for 30 min). After removing the surface oil using a micropipette, the supernatant was collected and freeze-dried at -50 ºC under vacuum (Labconco Freeze Dryer, USA). Obtained protein hydrolysates were named FPH_{2-45} (E/S ratio of 1:50 and time of 45 min), FPH_{2-90} (E/S ratio of 1:50 and time of 90 min), FPH_{2-180} (E/S ratio of 1:50 and time of 180 min), FPH_{4-45} (E/S ratio of 1:25 and time of 45 min), FPH_{4-90} (E/S ratio of 1:25 and time of 90 min) and FPH_{4-180} (E/S ratio of 1:25 and time of 180 min) and stored at -20 ºC until further analysis.

DPPH* radical scavenging activity

BHT and FPHs at different concentrations were prepared in distilled water. Then, 1 mL of sample solution was added to 1 mL of 0.1 mM DPPH solution (prepared in 95% ethanol) and the absorbance of the mixture was recorded at 517 nm after 20 min of incubation in dark. The control was prepared using 1 mL of distilled water instead of sample. DPPH radical scavenging activity was expressed as percentage of inhibition using the following equation: %DPPH radical scavenging activity $= [(Ac-As)/Ac] \times 100$. Where Ac is the absorbance of control and As is the absorbance of the sample. The effective concentration of sample required to inhibits 50% of the DPPH radical (IC_{50} value) was obtained by plotting a graph of concentration (X axis) versus percentage of inhibition (Y-axis) (Nikoo et al., 2014).

Fe²⁺ chelating activity

One mL of BHT or FPHs solution at different concentrations was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine and the final volume of the mixture was increased to 5 mL with addition of distilled water. After 20 min of incubation, the absorbance was recorded at 562 nm. For control sample, distilled water was used instead of the sample. Fe^{2+} chelating activity was calculated using the following formula. Fe^{2+} chelating activity (%) $= [(Ac-As)/Ac] \times 100$ where Ac is the absorbance of control and As is the absorbance of the sample. IC_{50} value was calculated from the plot of the chelating activity against the sample concentration (Nikoo et al., 2014).

Ferric reducing activity

A volume of 2 mL of protein hydrolysate (5 mg/mL) or BHT (0.5 mg/mL) was mixed with 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of 1% potassium ferricyanide. After incubation at 50 °C for 20 min, 2 mL of 10% Trichloroacetic acid (TCA) was added to the mixture. Following centrifugation at 3000 rpm for 10 min, 2 mL of the supernatant was mixed with 2mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. Then the optical absorbance was recorded at 700 nm (Nikoo et al., 2014).

ABTS** radical scavenging activity

The stock solution was prepared by mixing 7.4 mM $ABTS^+$ and 2.6 mM potassium persulfate solution (1:1) and left to incubate for 12 h at room temperature in the dark. Before the experiment, freshly prepared ABTS solution was diluted with methanol to reach an absorbance of 1.1 ± 0.02 at 734 nm. Then, 150 μL of FPHs or BHT at different concentrations was mixed with 2850 μL of ABTS solution, and after incubation at room temperature for 2 h, the optical absorbance was recorded. Control sample was prepared using 150 μL of distilled water instead of sample. ABTS scavenging activity was determined using the following formula. ABTS scavenging activity (%) $= [(Ac-As) / Ac \times 100]$; where Ac is the absorbance of control and As is the absorbance of the sample. IC_{50} value was determined from the plot of the scavenging activity against the sample concentration (Nikoo et al., 2014).

Hydroxyl radical scavenging activity

Briefly, 1, 10-phenanthroline solution (1.865 mM, 1 mL) and FPHs or BHT at different concentrations were added into a tube and mixed. Then, 1mL of the $FeSO_4$ solution (1.865 mM) was added to the mixture and the reaction was initiated by adding 1 mL of H_2O_2 (3% v/v). After incubation at 37 °C for 60 min in a water bath, the absorbance was recorded at 536 nm. Solution containing protein hydrolysis without hydrogen peroxide was considered as Blank and solution without protein hydrolysis was considered negative control. Hydroxyl radical scavenging activity was determined using the following formula. Hydroxyl radical scavenging activity (%) $= [(As-An)/(Ab-An)] \times 100$; where As is the absorbance of sample, An is the absorbance of the negative control and Ab is the absorbance of blank. IC_{50} value was determined from the plot of the scavenging activity against the sample concentration (Nikoo et al., 2014).

Evaluation of cytotoxic effects

MTT is a yellow water-soluble tetrazolium salt. It is reduced in the mitochondria of viable cells to generate

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*2,2-diphenyl-1-picrylhydrazyl

** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

a water-insoluble formazan salt. The MTT assay is a colorimetric method that evaluates the activity of cellular enzymes, in which yellow tetrazolium is converted to purple formazan. The assay is used to evaluate the proliferation of cells and the cytotoxic effects of drugs. $4T_1$ carcinoma cells line was purchased from National Cell Bank of Iran (Pasteur Institute., Tehran, Iran) and cultured in DMEM medium containing 10% FBS. After reaching around 80% confluence, they were detached by trypsin/EDTA and the number of cells was counted using a homocytometric lam. Then, 200 µL of suspension containing 15×10^3 cells was added to each well of a 96-well plate. In the next step, the cells were treated with FPHs/carboplatin at different concentration for 48 hours. After removing the medium of the well and washing by PBS, 60 µl of MMT solution in PBS was added to each well. The cells were then incubated at 37 °C in 5% CO_2 for 4 hours. After incubation, the medium was removed from the wells and 150 µL of DMSO was added to each well. The plate was then incubated for 30 minutes at 37 °C in the dark. Finally, the plates' absorbance was read at 570 nm using an ELISA reader. The percentage of cytotoxicity was calculated by the formula below:

$$\text{Percentage of cell cytotoxicity} = \left[1 - \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \right] \times 100$$

IC_{50} value determined from the plot of the scavenging activity against the sample concentration

Determination of molecular-weight distribution

The molecular weight distribution of the hydrolysate with the highest antioxidant activity was determined by gel permeation chromatography using HPLC system (Agilent 1100, USA). A TSK gel 2000 SWXL (300 × 7.8 mm) column (Tosoh, Tokyo, Japan) was equilibrated with acetonitrile: water (40:60, v/v) in the presence of 0.1% trifluoroacetic acid (TFA). The absorbance was monitored at 225 nm with flow rate of 0.5 mL/min. Cytochrome C (12384 Da), bacitracin (1422 Da), Gly-Gly-Try-Arg (451 Da), and Gly-Gly-Gly (189 Da) were used as protein molecular weight standards. The logarithm of molecular weight tested and the respective retention time was shown in a linear relationship. The equation $\sum (Mn \times Ai)/100$ was used to calculate the average molecular weight of sample (Guo *et al.*, 2013).

Determination of the amino acid composition

Amino acids were determined according to the AOAC method with some modifications. One hundred and twenty milligrams of the hydrolysate powder were digested with 8 mL of 6 M HCl at 110 °C for 22 hours under nitrogen atmosphere. After cooling, 4.8 mL of 10 M NaOH was added, the volume was made up to 25 mL with distilled water, then filtered through two layers of filter paper No. 40, and finally centrifuged at 10,000g for 10min. Amino acids were analyzed by using the reverse-phase high performance liquid chromatography (Agilent 1100 HPLC; Agilent Ltd., Palo Alto, CA, USA). Each sample (1 µL) was injected into a Zorbax, 80A C-18 column (column size: 4.0 × 250 mm, 5 µm particle size; Agilent, USA) at 40 °C with detection at 338nm. The mobile phase A was 7.35 mM/L of sodium acetate/triethylamine/ tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 using acetic acid, while the mobile phase B (pH 7.2) was 7.35 mM/L of sodium acetate/methanol/ acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as grams of amino acids per 100 g of protein (Guo *et al.*, 2013).

Statistical analysis

Data were analyzed using SPSS version 20. Analysis of Variance (ANOVA) followed by Duncan's test used to identify statistical differences between means. All data were presented as mean ± SD and p value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Figures 1-3 show the antioxidant activities of BHT and FPHs made by enzymatic hydrolysis of *L. klunzingeri* muscle protein. As shown BHT showed strong DPPH, ABTS and hydroxyl radicals scavenging activities (IC50 values of 0.047±0.01, 0.021±0.009 and 0.24±0.02 mg/mL respectively), Fe^{2+} chelating capacity (IC50 value of 0.057±0.01 mg/mL) and ferric reducing activity (optical absorbance of 1.16±0.09 at 700 nm wavelength). Protein hydrolysates obtained by enzymatic hydrolysis of *L. kludingeri* muscle using two concentration of papain at 45, 90, and 180 min, exhibited good scavenging activity on DPPH (IC_{50}=2.08-3.18 mg/mL), ABTS (IC_{50}=0.12-0.60 mg/mL), and hydroxyl (IC_{50}= 2.07-4.13 mg/mL) radicals, moderate chelating activities on Fe^{2+} (IC_{50}=2.12-12.60 mg/mL), and relatively poor ferric reducing activities (optical absorbance of 0.01-0.15 at 700 nm wavelength). With increasing the hydrolysis duration and the concentration of papain, the antioxidant activities of the FPHs in inhibiting the DPPH, ABTS, and hydroxyl radicals were increased, so that the highest inhibitory activity obtained for the FPH_{4-180} with IC_{50} values of 2.08 ± 0.13, 0.12 ± 0.01, and 2.07 ± 0.31 mg/mL,

respectively. Fe^{2+}- chelating activity of FPHs decreased with increasing the hydrolysis duration and FPH_{4-180} sample with the highest inhibitory activity on the ABTS, DPPH, and hydroxyl radicals showed the lowest Fe^{2+}-chelating activity ($IC_{50} = 12.60 \pm 0.02$). As shown in figure 3, the increase of hydrolysis time increased ferric reducing activity and therefore, FPHs obtained after 90 and 180 min of hydrolysis demonstrated better activities than sample obtained after 45 min of hydrolysis.

The analysis of the molecular weight distribution of the hydrlysate with the highest antioxidant activity (FPH_{4-180}) by using HPLC, showed that 95% of the peptides in this sample had a molecular weight of less than 1000 Da. 30.56% of peptides in this sample had molecular
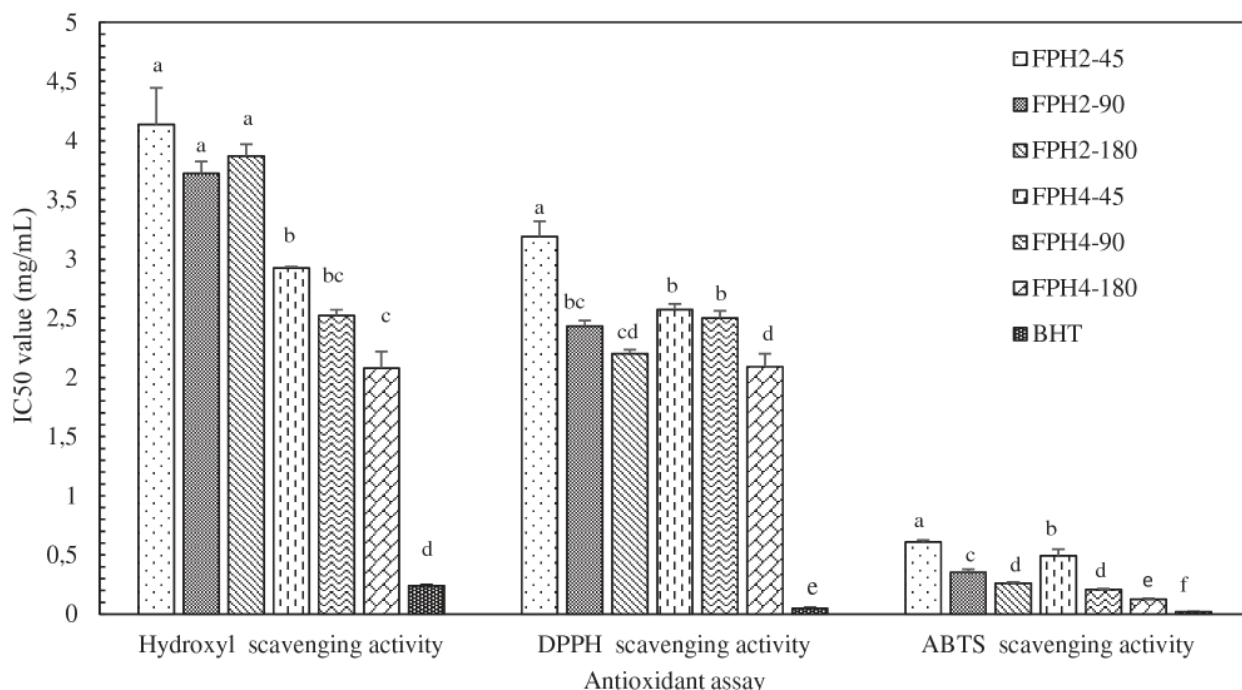

FIGURE 1 - The IC50 value of *Liza klunzingeri* muscle protein hydrolysates and BHT for DPPH, ABTS, and hydroxyl radicals scavenging activities. Different letters indicate statistically significant differences between antioxidant activities of samples (mean $\pm$ SD and $p<0.05$).
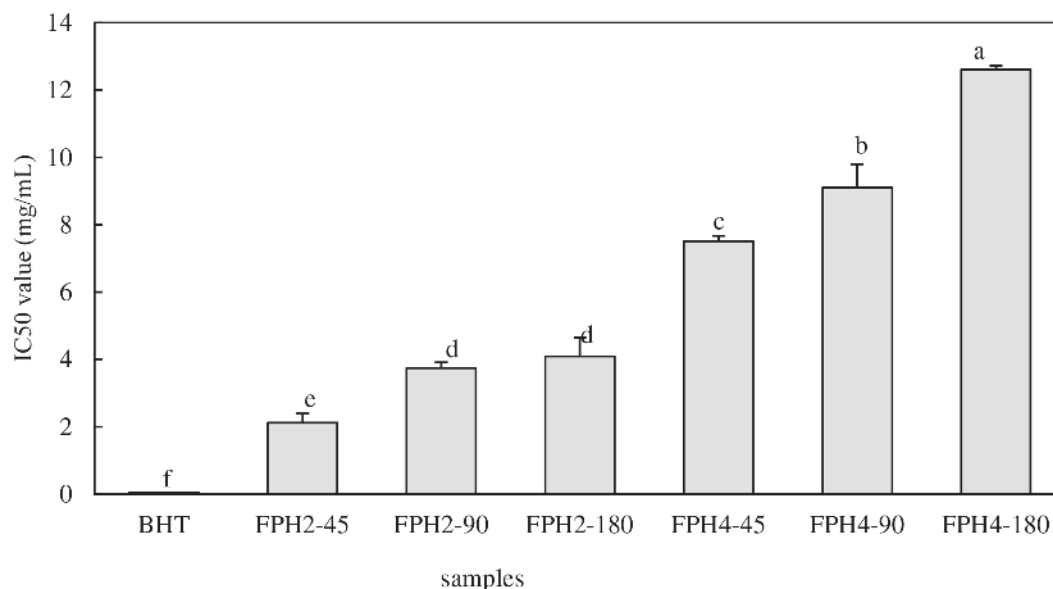

FIGURE 2 - The IC50 value of *Liza klunzingeri* muscle protein hydrolysates and BHT for Fe^{2+} chelating activity. Different letters indicate statistically significant differences between antioxidant activities of samples (mean $\pm$ SD and $p<0.05$).
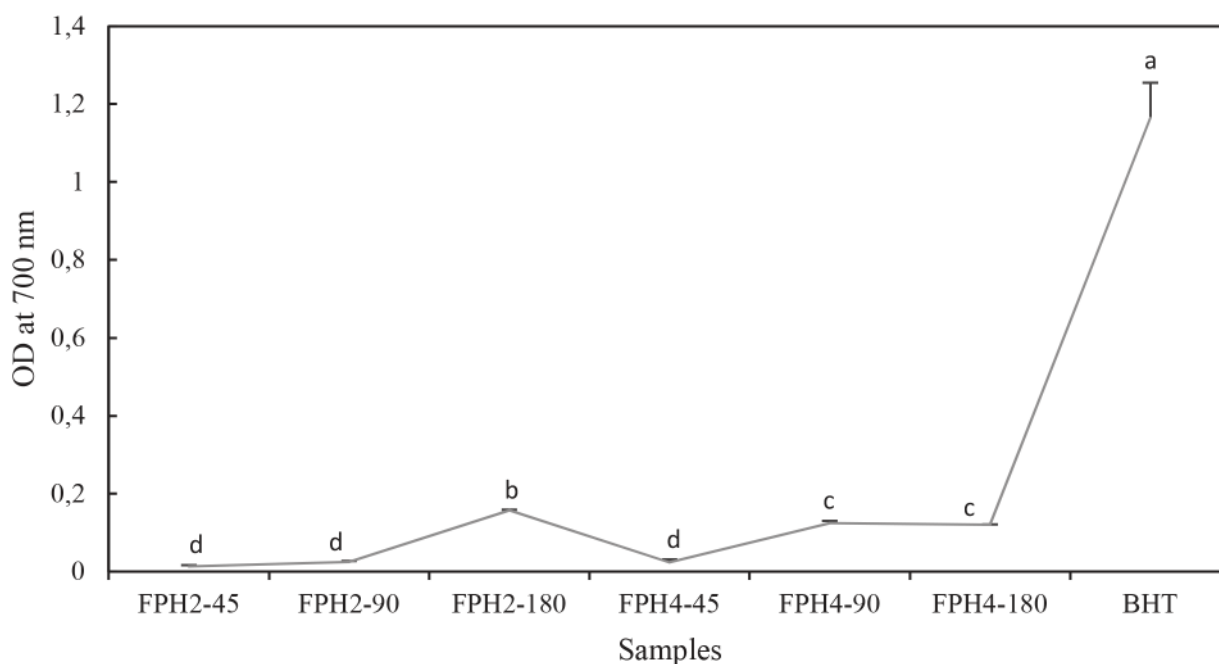
FIGURE 3 - The ferric reducing activity of *Liza klunzingeri* muscle protein hydrolysates and BHT. Different letters indicate statistically significant differences between ferric reducing activity of samples (mean $\pm$ SD and $p<0.05$)

weight of less than 180 Da, 47.26% had molecular weight of 180-500 Da, and 17.46% had molecular weight of 500-1000 Da (Figure 4).

It seems that the higher activity of FPH_{4-180} sample in inhibition of DPPH, ABTS, and hydroxyl radicals is due to the presence of low-molecular-weight peptides, which increased with increasing hydrolysis duration. Several studies have suggested that high degrees of hydrolysis and low molecular weight have a positive correlation

with the DPPH and ABTS radical scavenging activity (Bougatef *et al.,* 2010; Liu *et al.*, 2010; Phanturat *et al.*, 2010; Raghavan, Kristinsson, 2008), although some studies have reported an inverse relationship (Alemán *et al.*, 2011a; Theodore, Raghavan, Kristinsson, 2008).

Also, in the present study, the Fe^{2+} chelating activities of the FPH_S showed the opposite trend and FPH_{4-180} had the lowest Fe^{2+}-chelating activity. In a study by Pownall, Udenigwe and Aluko (2010), the pea seed
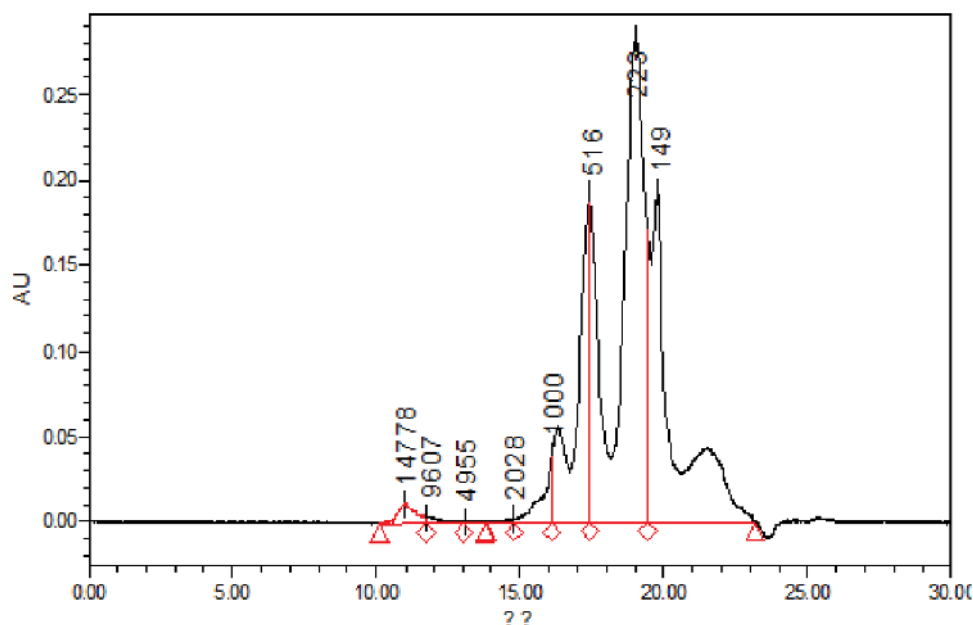

FIGURE 4 - Molecular weight distribution of *Liza klunzingeri* protein hydrolysate with the highest antioxidant activity.

protein hydrolysate, which showed the highest Fe^{2+}-chelating activity, had a poor inhibitory effect on the ABTS, DPPH, hydroxyl, and hydrogen peroxide radicals, which is consistent with our results (Pownall, Udenigwe, Aluko, 2010). Alemán *et al.* (2011b) argued that squid gelatin hydrolysates with a higher degree of hydrolysis and a lower molecular weight, exhibited better Fe^{2+}-chelating activity (Alemán *et al.*, 2011b), while Bamdad, Wu and Chen (2011) reported that peptides with a higher molecular weight exhibited higher Fe^{2+}-chelating activity, which is due to the trapping of iron ions in the peptide chain (Bamdad, Wu, Chen, 2011).

Furthermore, we found that the ferric reducing activities of FPHs increased with the increasing the time of hydrolysis and therefore breakdown of large peptides into smaller peptide units. Contradictory results have been reported regarding molecular weight relationship with ferric reducing activity, some of which, in agreement with the present study, have shown an inverse correlation between ferric reducing activity and molecular weight (Alemán *et al.*, 2011a), and others have indicated a positive correlation (Theodore *et al.*, 2008).

The inconsistencies in the findings of various studies suggest that molecular weight is not the main determinant of the antioxidant activity of the protein hydrolysate and peptide samples. It has been reported that the antioxidant properties of the bioactive peptides depends on their size of peptides and also amino acid sequences, which are influenced by the source of substrate protein, type of enzyme used, enzyme to substrate ratio and hydrolysis conditions (temperature, pH and time) (Harnedy, FitzGerald, 2012).

In this study, the most abundant amino acids in $FPH_4\text{-}_{180}$ sample were serine (9.593%), tyrosine (8.43%), cysteine (7.197%), valine (6.60%), histidine (5.81%), and glutamine (4.914%) (Table I). It is reported that aromatic amino acids (phenylalanine, tryptophan and tyrosine) donate the electron to free radicals and make them stable molecules (Sarmadi, Ismail, 2010). Amino acids, such as histidine, leucine, tyrosine, methionine, and cysteine, neutralize free radicals by donating proton (Mendis *et al.*, 2005), fat-soluble free radicals (peroxyl radicals) that are produced throughout the oxidation of unsaturated fatty acids are neutralized by hydrophobic amino acids such as leucine, valine, alanine, and proline (Kim, Mendis, 2006). Thus, it can be argued that natural protein hydrolysates may have inhibitory effects on several types of free radicals due to the presence of various amino acids, while purified peptide from a protein hydrolysate that contains fewer types of amino acids may exert low inhibitory effect on some free radicals. In the study on fractions

derived from Cod protein hydrolysates, it was observed that the isolation of different fractions with strong DPPH scavenging effect resulted in a decrease of ferric reducing activity, which was due to an increase in the ratio of positively charged amino acids to sulfur-containing amino acids (Girgih *et al.*, 2015).

TABLE I - Amino acid composition of Liza klunzingeri protein hydrolysates with the highest antioxidant activity

Amino acid	g/100 g pr
Aspartic Acid	2.93908
Glutamic Acid	4.91412
Serine	9.50344
Histidine	5.81917
Glycine	1.45979
Threonine	1.08553
Arginine	1.94127
Alanin	1.81932
Tyrosine	8.34432
Cysteine	7.01992
Methionine	1.87524
Valine	6.16082
Phenylalanine	1.11977
Isoleucine	1.45958
Leucine	2.25984
Lysin	2.64536
Proline	2.64536

As shown in Figure 5, the FPHs obtained from enzymatic hydrolysis of *L. klunzingeri* showed significant cytotoxic activities (IC_{50}=1.62-2.61 mg/mL) on $4T_1$ breast cancer cell line. The cytotoxic activities of samples decreased with increasing the hydrolysis duration and $FPH_4\text{-}_{45}$ sample that was hydrolysed for a shorter period showed the highest cytotoxic activity (IC_{50} = 1.62 ± 0.10 mg/mL, Figure 5). In the study of Picot *et al.* (2006) the cytotoxic effects of protein hydrolysates of 18 fish species were determined on MCF-7/6 and MDA-MB-231 cancer cells, and the highest cytotoxic effect (up to 40%) was exhibited by the Cod protein hydrolysate (1 mg/kg, 72 h) on the MCF-7 cell line (Picot *et al.*, 2006). Tuna muscle protein hydrolysate also showed a significant inhibitory effect on the MCF-7 cell line, and the highest inhibitory activity was obtained for the fraction with 390-1000 Da molecular weight (Hsu, Li-Chan, Jao, 2011). In a study, fraction with low MW peptides (<3 kDa) isolated from Loach
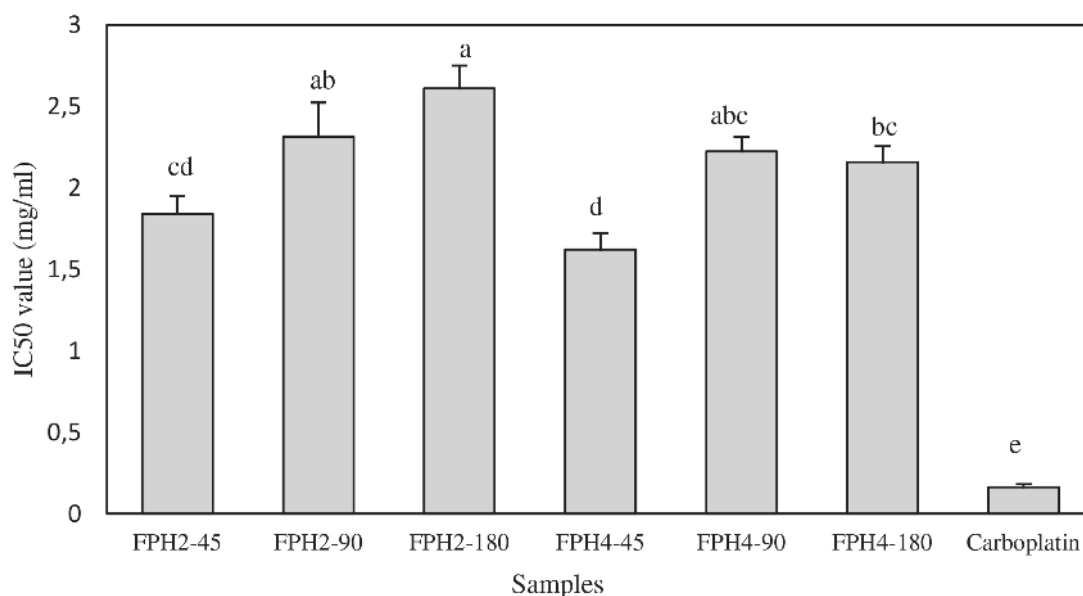
FIGURE 5 - The IC_{50} value of *Liza klunzingeri* muscle protein hydrolysates and carboplatin against $4T_1$ cancer cell line. Different letters indicate statistically significant differences between antioxidant activities of protein hydrolysate samples (mean ± SD and $p<0.05$).

protein hydrolysate showed better cytotoxicity than fractions with high MW peptides (3-5 kDa, 5-10 kDa and > kDa) (Zhao, Liu, Regenstein, 2011). However, some high MW peptides derived from buckwheat seeds (approximately 4 kDa) (Leung, Ng, 2007) and soybean protein hydrolysate (> 10 kDa) were also reported to show significant cytotoxicity (Marcela *et al*., 2016). Therefore, some researchers have claimed that the cytotoxicity of the peptides depends not only on the chain length, but also amino acid sequences, which are influenced by the source of substrate protein, type and amount of enzyme used, and hydrolysis conditions (Alemán *et al*., 2011b; Picot *et al*., 2006). In the present study, 64.72% of peptide in FPH_{4-180} sample had molecular weight distribution less than 500 Da. It seems that the molecular weight of peptides and therefore the cytotoxic activity were reduced compared to the samples hydrolysed at shorter time.

Table II shows the proximate composition of *L. klunzingeri* muscle and protein hydrolysate (with highest antioxidant activity, FPH_{4}-$_{180}$ sample). The protein contents of *L. klunzingeri* muscle and protein hydrolysate were 87.84 ± 2.85% and 22.45 ± 3.39%. This indicates that

protein hydrolysate obtained from enzymatic hydrolysis of *L. klunzingeri* muscle has a high nutritive value due to the presence of high level of protein/amino acids.

In this study, however, FPHs exhibited much lower antioxidant and cytotoxic activities than do BHT and carboplatin, but they are safer to eat, causing fewer adverse effects and provide the body a rich source of high-quality protein. BHT is a synthetic antioxidant commonly used in products such as food, cosmetics and pharmaceutics, but it might exert some toxic side effects on body's tissues, leading to the development of cancer (Witschi, 1986). It was also reported that the oral administration of BHT could induce oxidative stress by interfering with oxidative-antioxidant balance (Faine *et al*., 2006). Carboplatin is a potent chemotherapy medication used to treat a number type of cancer but, it often causes specific side effects such as anemia, nausea, electrolyte problems, allergic reactions and increased risk of another cancer (Tothilla *et al*., 1992). So there is a growing trend to replace these synthetic compounds with natural ones to prevent or alleviate oxidative stress and associated diseases (Hsu, Li-Chan, Jao, 2011).

TABLE II - The moisture, total protein, fat, and ash contents of *Liza klunzingeri* muscle and protein hydrolysate

Fat	Ash	Protein	Moisture	Samples
		Mean±SD		
0.77±0.46	9.52±0.83	87.84±2.85	1.87±1.72	P4-180
2.21±0.53	2.00±0.42	22.46±3.41	73.36±3.90	Protein

Based on the findings of the present study, the protein hydrolysates of *L. klunzingeri* muscle exhibit significant antioxidant and cytotoxic properties *in vitro*. Besides, it has a high nutritional value because of its valuable content of protein and essential amino acid. However, in the body, peptides present in the protein hydrolysates may be metabolized due to enzymatic and digestive processes, and their structure and activates may be altered; therefore, it is recommended that the efficiency of protein hydrolysates obtained from L. *klunzingeri* muscle be investigated and confirmed in animal models, before suggesting them as complementary and health-promoting compounds. It is also recommended to optimize the hydrolysis conditions to reach the sample with maximum efficiency *in vitro*, and to use the sample with the highest antioxidant or cytotoxic activity in subsequent investigations to observe the maximum efficiency in animal models. After obtaining promising results in animal and human studies and the necessary approvals, antioxidant and cytotoxic protein hydrolysate can be commercially produced and used as functional compounds.

CONCLUSION

Production of protein hydrolysates from Klunzinger's mullet represented an alternative way of upgrading this species. The antioxidant and cytotoxic properties of klunzinger's mullet protein hydrolysates showed that these hydrolysates present potential as a functional food ingredient or as natural food supplement.

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