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Original Article

# CHARACTERIZATION OF ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF INDIAN SALMON (*ELEUTHERONEMA TETRADACTYLUM*) PROTEIN HYDROLYSATES

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**ABSTRACT**

**Objective:** The main aim of the present research was to evaluate the antioxidant and antiproliferative activity of Indian salmon (*Eleutheronema tetradactylum*) protein hydrolysates by pepsin and trypsin enzyme using *In vitro* gastrointestinal digestion procedure.

**Methods:** Indian salmon protein hydrolysates (ISPH) were fractionated to obtain fraction I (FI), fraction II (FII) and fraction III (FIII) of peptides with different molecular weights (MW). Further, the antioxidant activity of ISPHs was evaluated by DPPH radical scavenging, metal chelating, reducing power and lipid peroxidation assays. Moreover, *In vitro* antiproliferative activity of ISPHs was assessed against breast cancer cell lines MCF-7. The amino acid contents of the bioactive peptides were also determined to find the correlation between the activity of peptides and their amino acid contents.

**Results:** All bioactive peptides showed dose-dependent antioxidant activities. The highest antioxidant activity was measured in FII which was able to quench higher levels of free radicals. In the measurement of the antiproliferative capacity of peptides, they revealed nearly similar activities at low concentration. However, the cytotoxicity of peptides was significantly increased at the high dose in which only 43.9±1.8% to 65.7±1.6% cell proliferation occurred. The results showed an absence of correlation between MW and activity of peptides since the most potent bioactive peptides in our study had MWs of 1 to 3 kDa. However, hydrophobicity and presence of special amino acids like arginine and histidine is affected the activity of peptides.

**Conclusion:** Consequently, Indian salmon protein hydrolysates were identified as good sources of antioxidant and antiproliferative peptides which could confer both nutritional and functional properties in the food industry.

**Keywords:** Indian salmon, Protein hydrolysates, Antioxidant, Antiproliferative, Amino acid

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## INTRODUCTION

Oxygen free radicals (ROS) are products of oxidative metabolism in aerobic organisms during respiration. ROS are highly unstable and can damage macromolecules such as proteins, lipids and nucleic acids [1]. Many studies have shown the implication of reactive radicals in the occurrence of serious health problems including inflammatory diseases, vascular dysfunction, cancer, diabetes, aging, neurological disorders and cardiovascular diseases [2]. Furthermore, free radicals can also lead to lipid peroxidation in food products which is characterized by undesirable odor, off flavor and subsequently production of toxic compounds. However, the most effective way to eliminate free radicals is the use of antioxidants. These compounds minimize tissue damages by preventing the formation of radicals, scavenging them or by promoting their decomposition [3].

Although many synthetic (BHA and BHT) and natural (a-tocopherol and Vitamin C) antioxidants are used to prevent or retard the effects of free radicals, the use of these compounds is restricted due to various undesirable health effects [4]. Therefore, characterization of antioxidative peptides from natural sources especially food hydrolysates is of pivotal interest for pharmaceutical scientists. The activity of biopeptides mainly consisting of 3 to 20 amino acid residues depends on their amino acid composition, the source of protein, the degree of hydrolysis and peptide structure [1]. Recent reports have revealed the antioxidant, antidiabetic [5], immune-regulatory [6], antiproliferative [7] and antihypertensive [8] activities of fish protein hydrolyses (FPH).

Cancer is one of the major causes of death in humans. Conventional treatments such as surgical removal and chemotherapy have not yet improved the survival rate over the past two decades [9]. For this reason, it has become necessary to find more effective and less toxic antitumor drugs [10]. FPHs have been shown to possess potential for nutritional or pharmaceutical applications [11]. However, the antiproliferative activity of peptides derived from FPH is rarely studied. Earlier, Leung and Ng [12] reported the antiproliferative activity of peptides obtained from buckwheat protein hydrolysates against leukemia, breast cancer, liver embryonic and liver cancers cells. Lunasin, a bioactive peptide with MW of about 5.45 kDa, was isolated from barley and soy protein hydrolysates. This peptide was found to inhibit the action of oncogenes and carcinogens in skin cancer mouse model [13].

Therefore, the objective of this study was to determine the antioxidative and antiproliferative potentials of Indian salmon protein hydrolysates obtained by *in vitro* gastrointestinal digestion procedure. The antioxidant activity of peptide fractions was evaluated by DPPH radical scavenging, inhibition of linoleic autoxidation, reducing power, and metal chelating assays. Furthermore, the antiproliferative property of peptides was characterized by breast cancer cell lines MCF-7. Eventually, the amino acid composition of bioactive peptides was determined to find the possible correlation of their amino acid contents with antioxidant and antiproliferative activities.

## MATERIALS AND METHODS

### Reagents and chemicals

DPPH, ammonium thiocyanate, α-linoleic acid, and ultrafiltration membrane discs were bought from Sigma-Aldrich trading Co., Ltd (Bangalore, India). Proteases for enzymatic hydrolysis (pepsin and trypsin) were obtained from Sigma-Aldrich Ltd, MO, USA. MCF-7 cell line of human breast cancer was purchased from National Centre for Cell Science (NCCS), Pune, India. DMEM media and FBS were also obtained from HIMEDIA and GIBCO (India), respectively. All other chemicals used were analytical grade.

### Sample preparation

Indian salmon (*Eleutheronema tetradactylum*) fish was obtained from Mysore (Mysore, India) market. The samples were placed in polyethylene bags and kept in ice. They were transported to the

laboratory within 30 min and stored at −80 °C until further use. Fish muscles were carefully separated and prepared for hydrolysis

**Proximate composition**

Moisture and ash contents of Indian salmon fish muscle were determined according to AOAC [14], methods 930.15 and 942.05, respectively. The crude protein (N × 6.25) was measured using Kjeldahl method [15]. Lipid content was found through Bligh and Dyer method [16].

**Preparation of Indian salmon protein hydrolysates**

Indian salmon protein hydrolysates (ISPH) were prepared following the method of [17] with some modifications. Fifty gram of Indian salmon (*Eleutheronema tetradactylum*) muscle was homogenized with 100 ml of distilled water and incubated at 25 °C for 20 min prior to enzymatic hydrolysis. The pre-incubated proteins were then hydrolyzed by pepsin (8000 units/mg) for about 1.5 h by vigorous stirring at 37 °C and pH 2.0. This was followed by further digestion of Indian salmon proteins with trypsin (1000 units/mg) at pH 6.5 and 37 °C. Enzymatic hydrolysis was stopped by heating the mixture for 10 min in boiling water bath. Hydrolysates were then centrifuged at 12,000×g for 15 min. The supernatant was lyophilized and stored at-20 °C until further use.

**Purification of peptides by ultrafiltration**

An amount of 3 liters of ISPH solution was filtered through an ultrafiltration unit (Millipore Corporation, Bedford, USA) with molecular cutoffs of 5 kDa and 10 kDa to obtain 3 fractions of varying molecular weights. The molecular weight of resulted fractions was<5 kDa, 5-10 kDa and>10 kDa.

The fraction with highest antioxidant and antiproliferative activity (ISPHI) was further fractioned with a molecular cut-off of 1 kDa and 3 kDa to attain peptides with MW ranges of<1 kDa, 1-3 kDa and 3-5 kDa. The fractions were lyophilized and stored at-20 °C for further use.

**Amino acid composition of ISPHs**

ISPHs were hydrolyzed with 0.5 ml of HCl (6 N) at 110 °C for 24 h. HPLC detection of amino acids was done by derivatization with phenyl isothiocyanate. Tryptophan was also determined using alkaline hydrolysis [18]. HPLC (Waters, Milliford, Ma, USA, equipped with PICO-TAG column) detection of amino acids was done following the method [19] with slight modification.

**Antioxidant activity of protein hydrolysates**

**DPPH radical scavenging activity**

DPPH assay was performed according to the method [20] with slight modification. Two milliliters of samples with varying concentration (1, 2, 5 mg/ml) were added to 2 ml of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken and incubated at dark for 30 min at room temperature. Scavenging activity was found by measuring absorbance at 517 nm. BHA was used as positive control. The scavenging activity was calculated by the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{absorbance of blank} - \text{absorbance of sample})}{\text{absorbance of blank}} \times 100$$

**Determination of metal chelating activity**

The ability of ISPHs to chelate ferrous ions was assessed using the method of Decker and Welch [21]. A total volume of 0.5 ml of sample with different concentrations (1, 3, 5 mg/ml) was mixed with 1.5 ml of distilled water. Later, 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of ferrozine were added. The mixture was allowed to stand at room temperature for 20 min and the absorbance was read at 562 nm. EDTA was used as the reference. The $Fe^{2+}$chelating activity was calculated using the formula:

$$\text{Chelating activity (\%)} = \left[1 - \frac{A_{562}\text{ of sapmle}}{A_{562}\text{ of blank}}\right] \times 100$$

**Reducing power**

The reducing power of ISPHs was measured according to the method of Oyaizu [22]. To 2 ml of the sample of varying concentrations (1, 3, 5 mg/ml), a volume of 2 ml 0.2 M phosphate buffer pH 6.6 and 2 ml of potassium ferricyanate was added and incubated at 50 °C for 20 min. Then, 2 ml of 10% TCA was added to each mixture. Two milliliters of this mixture was mixed with 2 ml of distilled water and 0.4 ml of 0.1% ferric chloride. The mixture was incubated at room temperature for 10 min and the absorbance was measured at 700 nm. Ascorbic acid was used as positive control.

**Inhibition of linoleic acid autoxidation**

The antioxidative activity of protein hydrolysates using α-linoleic acid model system was measured following the method described by Osawa and Namiki [23]. One milligram of the sample was dissolved in 2.5 ml of phosphate buffer pH 7.0 (50 mM) and added into 0.05 ml of 50 mM linoleic acid in 2.5 ml of 95% ethanol and mixed well.

The volume of the mixture was made up to 6.25 ml using distilled water and incubated in dark at 40±2 °C. The degree of linoleic oxidation was measured at 24 h intervals following the method of Dong *et al.* [24] using ferric thiocyante and measuring the absorbance at 500 nm. The activity of hydrolysates was compared with α-tocopherol and control.

**Cell culture**

Human breast cancer MCF-7 cell lines (NCCS, Pune, India) were used for cell culture. The cells were cultured in DMEM medium supplied with 10% heat-inactivated FBS and 1% Pen-strep antibiotic (Sigma) at 37 °C in a 5% $CO_2$ enriched humidified incubator.

**MTT assay**

MTT assay was performed as described by Mosmann [25]. Based on the preliminary observations, MCF-7 cells in the exponential phase were seeded onto 96 well plates (10 x $10^4$ cells/well), allowed to adhere for 24 h and treated with various concentrations of ISPHs ranging from 50 to 200 µg/ml. The medium was removed, and the cells were washed with PBS. A volume of 100 µl of MTT reagent (3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) (5 mg/ml) was added to each well. After 4 h of incubation, the solution was removed, and 100 µl of DMSO was added to each well. The cells were incubated for 10 min and the absorbance was read at 540 nm on an ELISA reader.

**Statistics**

All analytical determinations were performed at least in triplicate. Antioxidant and antiproliferative data are given as mean data±standard deviation.

**RESULTS AND DISCUSSION**

**Proximate composition**

The proximate composition of Indian salmon fish muscle was evaluated to determine the crude protein contents (table 1). The protein contents measured in our study accounted for 19.21±2.3%, which was higher than that of horse mackerel [1] and confirmed the nutritional value of studied fish species. Our results were in accordance with the earlier studies stating that the protein contents in marine fish species range from 8% to 21% [26].

**Table 1: Proximate composition of Indian salmon muscle including moisture, ash, protein and lipid contents, Data are shown as mean±SD**

| Proximate composition | Percentage (%) |
|---|---|
| Moisture | 76.34±1.8 |
| Ash | 1.7±0.3 |
| Crude protein | 19.21±2.3 |
| Lipid | 2.1±0.6 |

Values are expressed as±SD, n = 3.

**Fractionation of protein hydrolysates**

In order to obtain peptides fractions with different MWs, the hydrolysates were fractionated through 5 kDa and 10 kDa membranes. As a result, three fractions namely ISPHI (MW<5kDa), ISPHII (MW 5-10 kDa) and ISPHIII (MW>10 kDa) were obtained. The fraction with highest antioxidant and antiproliferative activity (ISPHI) was further separated using 1 kDa and 3 kDa membrane discs to attain fractions namely; FI (MW<1 kDa), FII (MW 1-3 kDa) and FIII (MW 3-5 kDa). The resulted fractions accounted for 21%, 42% and 15% of total obtained peptides, respectively.

**Antioxidant properties of ISPHs**

**DPPH radical scavenging activity**

The reaction of superoxide radicals with Biomolecules in body damages cellular components including proteins, enzymes, DNA and membrane lipids. These effects have to be overcome by the body through scavenging activity [5]. The antioxidant activity of ISPHs was examined through using DPPH radical scavenging assay. Fig. 1A depicts the DPPH radical scavenging activity of ISPHs, unfractionated protein hydrolysate (UFPH) and BHA as the positive control at different concentrations. The overall picture of the graph shows a concentration-dependent radical scavenging activity of peptide fractions with greater DPPH activity at the higher concentration of peptides. UFPH, which includes peptides of varying molecular weights and amino acid compositions, showed the lowest DPPH scavenging activity at all concentrations. BHA as the positive control scavenged 96.3% of DPPH radicals in 5 mg/ml. As it is seen in fig. 1A, all ISPHs except FII showed nearly close scavenging activities at low concentration (1 mg/ml). However, at higher peptide doses (3 and 5 mg/ml) all fractions were able to scavenge greater levels of free radicals. DPPH scavenging potential of fractionates at 5 mg/ml ranged from 79.7±1.1% (FI) to 88.1±0.7% (FII). This was followed by 76.8±1.3% scavenging activity of ISPHI with MWs lower than 5 kDa. In overall, FII (MW 1-3 kDa) was identified as the most potent fraction and capable of inhibiting radical mediated peroxidation. Moreover, the molecular weight of bioactive peptides in our study was similar to that of tuna dark muscle hydrolysates [27].

A possible reason for the high antioxidant potential of FII fraction might be variation in amino acid constituents of its peptides. Amino acid composition of peptides is believed to impact their DPPH radical scavenging activity. It is presumed that high hydrophobic amino acid (HAA) in peptides contributes structural properties that increase their interaction with membrane lipid bilayer. This interaction occurs through hydrophobic interactions and facilitates peptide entry into target organs [27]. As it is seen in the table (2), a good proportion of HAAs were found in Indian salmon protein hydrolysates that ranged from 38.49% (FI) to 40.49% (FII) of total amino acids. Few amino acids including His and Cys are commonly believed to enhance the radical scavenging activity of peptides. Therefore, higher Cys and sulfhydryl residues increase scavenging potential by donating sulfur hydrogen to electron deficient atoms [1]. Indeed, ISPHs displayed good quantities of these two amino acids with the highest measures in FII peptides that accounted for 3.55% (His) and 1.02% (Cys) of total amino acids (table 2).

Non-polar aliphatic amino acids such as Leu and Ala facilitate inhibition of radical mediated peroxidation through reacting with hydrophobic PUFA [1]. Accordingly, the highest amounts of Leu and Ala were observed in FII that constituted 8.6% and 5.39% of total amino acids, respectively. Moreover, aromatic amino acids such as Trp and Tyr had been proved to inhibit generation of lipid oxidation byproduct through free radical scavenging [28]. The amount of these amino acids accounted for 4.73% and 3.54% in FII fraction, respectively.

**Metal chelating activity**

Transition metals such as $Fe^{2+}$ are involved in the generation of reactive oxygen species and lipid peroxidation that subsequently result in many diseases. Therefore, metal chelating assay estimates the ability of peptides in chelating these metal ions and preventing their negative health effects. Metal chelating capacity of UFPH, fractionated hydrolysates and EDTA (The positive control) are presented in fig. 1B. As a result, UFPH exhibited the lowest chelating activity compared to other peptides. This indicates the effect of fractionation and molecular weight on the metal chelating capacity of peptides which was in concordance with earlier studies [5]. In fact, peptide cleavage increases the $Fe^{2+}$binding sites by enhancing the number of carboxyl and amino groups. All peptide fractions were able to convert positively $Fe^{2+}$to $Fe^{3+}$ions at different concentrations. As an overall trend, the metal chelating activity of peptides was dose-dependent and increased with increase in concentration, which was on a par with earlier reports [29]. However, the highest activity was measured in FII fraction having peptides with MW of 1 to 3 kDa. This was followed by FIII, FI and ISPHI at last. At high concentration (5 mg/ml) the fractionated peptides could chelate 79.8% (FI) to 88.3% (FII) of metal ions that was considerably significant when compared to UFPHs. Moreover, EDTA as the positive control could successfully chelate 99.1% of $Fe^{2+}$ions.

Enzymatic hydrolysis of proteins can directly impact their chelating activity through producing smaller peptides and free amino acids. Other parameters including size and amino acid composition may affect chelation of metal ions too [1]. In addition, Saidi *et al.* [27] reported the direct effect of His concentration on chelating capacity through increasing the ionic interaction of peptides with iron. Similarly, a higher amount of His was observed in FII fraction (3.55%) with the highest iron chelating capacity. It has also been shown that the level of Glu has a direct correlation with metal chelating activity of peptides and affects it through electrostatic and ionic interactions with iron [27]. In overall, ISPHs could successfully chelate high proportion of $Fe^{2+}$ions and showed great antioxidant activities.

**Reducing power**

Reducing power could be defined as the ability of an antioxidant to reduce a compound or donate electrons to deficient electron systems [27]. A direct relationship between antioxidant activity and reducing the power of peptides has been suggested in earlier studies (24). Therefore, the reducing capacity of ISPHs at different peptide concentrations (1, 3, 5 mg/ml) was assessed using their ability to reduce potassium ferricyanate.

Fig. 1C shows dose-dependent reducing the capacity of ISPHs which increases at higher peptide concentrations. Vitamin C as the positive control showed the greatest reducing ability with the highest absorbance at 700 nm. In contrast, UFPH exhibited the lowest ability in $Fe^{3+}$reduction in comparison to fractionated peptides. FII fraction with MW of 1 to 3 kDa displayed a significant $Fe^{3+}$reducing capability (1.74±0.05) at the concentration of 5 mg/ml, followed by very close activity of FIII (1.52±0.06), FI (1.51±0.81) and ISPHI (1.47±0.28) fractions. The results revealed that the reducing power of peptides was independent of their molecular weight since there was no correlation between their reducing capacity and molecular weights.

Amino acid composition and peptide sequence are generally believed to affect the antioxidant activity of protein hydrolysates. However, the hydrolysis condition including digestive enzymes, substrate/enzyme ratio, pH, temperature and reaction time could alter the amino acid composition of resulting peptides [28]. In addition, HAA is considered as factors increasing the antioxidant activity of peptides. A possible reason for this could be increased the solubility of peptides in lipid targets which promotes their interaction with free radical species.

Moreover, positively charged amino acids including His and Arg that are believed to enhance the antioxidant activity [27]. These two amino acids were highly found in studied three fractions. The highest quantities of His and Arg were measured in FII fraction that accounted for 3.55% and 3.87% of total amino acids, respectively. Generally, a higher concentration of carboxyl and amino side chains produced upon hydrolysis enhances the reducing capability of peptides. Indeed, the presence of indolic, phenolic and imidazole side chains as hydrogen donors are believed to facilitate reduction [30]. The results showed that ISPHs had strong reductive capacities to donate electron or hydrogen to deficient electron systems.

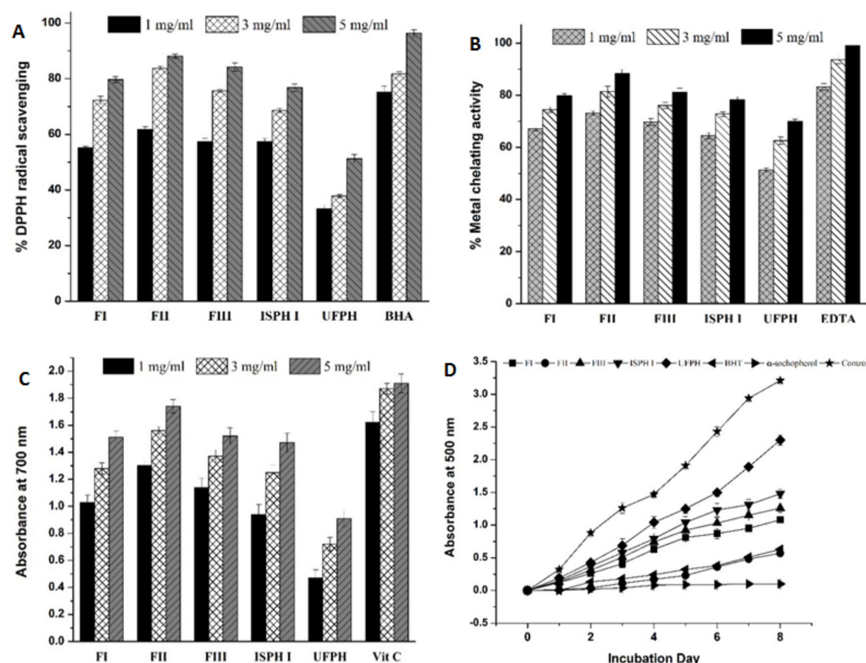


**Fig. 1: A) DPPH free radical scavenging activity of ISPHs, UFPH at different concentrations and BHA was used as positive control. B) Metal chelating activity of ISPHs and UFPH at different concentrations. EDTA was used as positive control. C) Reducing power of ISPHS and UFPH at different concentrations. Vitamin C was used as positive control. D) Inhibition of lipid peroxidation by ISPHs and UFPH in 8 d of incubation. BHT and α-tocopherol were used as positive control. Higher absorbance at 500 nm shows lower inhibitory effect. Each value is expressed as the mean±standard deviation (n = 3)**

**Lipid peroxidation assay**

Lipid peroxidation as a consequence of free radical activity in food oil and lipids is believed to proceeds through radical mediated abstraction of hydrogen atoms in PUFA [28]. Peroxidation of lipids leads to off flavor, undesirable odor and subsequently toxicity to mammalian cells [1]. In addition to food rancidity, free radicals in the body could result in conditions including ocular, cardiovascular, neurological and pulmonary diseases, cancer, rheumatoid arthritis and nephropathy [2]. Therefore, a growing interest in the identification of an antioxidant compound from food materials has been developed. Accordingly, the antioxidant activity of ISPHs was characterized by evaluating their ability to inhibit linoleic acid peroxidation using thiocyanate method and comparing their activity with that of BHT and α-tocopherol.

Fig. 1D depicts inhibition of lipid peroxidation by ISPHs in an incubation period of 8 d. It has to be noted that the inhibitory activity of peptides is indirectly related to their absorbance at 500 nm, i.e., higher the absorbance at 500 nm, lower the linoleic acid inhibitory activity. As it is shown in fig. 1D, UFPHs exhibited the lowest lipid peroxidation inhibitory activity compared to fractionated peptides. The highest inhibitory activity was seen in FII (MW 1-3 kDa) as the most potent and effective inhibitor of linoleic oxidation among all fractions which was greater than the natural antioxidant BHT and very close to α-tocopherol. This was followed by FI (MW<1 kDa), ISPHI (MW<5 kDa) and FIII (MW 3-5 kDa) fractions of varying molecular weights.

In earlier studies, peptides with MW of 1.8 kDa in hoki [31] and 1 kDa in Alaska pollak [32] exhibited high lipid peroxidation inhibitory effect. However, in our study, the bioactive peptides contained MWs between 1 to 3 kDa. Unexpectedly, ISPHI exhibited a higher degree of linoleic acid inhibition than FIII peptides. However, this may indicate that inhibition of linoleic acid peroxidation in ISPHI fraction is due to the activity of peptides with MWs lower than 3 kDa. Control sample which lacked peptides showed the highest rate of lipid peroxidation after incubation for 8 d. This was in contrast with α-tocopherol which displayed the lowest absorbance at 500 nm and could significantly inhibit peroxidation of linoleic acid during 8 d.

However, Saidi *et al.* [27] stated the role of HAA in inhibition of linoleic acid autoxidation by enhancing the solubility of peptides in the lipid phase. In fact, the property of HAA including Val and Leu in positive inhibition of lipid peroxidation could be attributed to their ability in facilitating a better interaction with lipid-derived radicals [27]. Similarly, a good proportion of hydrophobic amino acids were observed in ISPHs with the highest amount in FII fraction. Accordingly, Leu and Val were highly found in FII fraction, constituting 8.6% and 6.18% of total amino acids, respectively (table 2).

**Antiproliferative activity of peptides against MCF-7 cells**

The antiproliferative activity of ISPHs at different concentrations against MCF-7 breast cancer cell line is depicted in fig. 2. As it is seen in fig. 2, ISPHs showed remarkable antiproliferative activity and cytotoxicity against MCF-7 cells at higher concentrations (10 and 20 μg/ml). It is noteworthy that the peptides showed almost similar inhibitory effects at the low concentration of 5 μg/ml, in which 79.3±1.1 to 83.5±3.1% cell proliferation was observed in MCF-7 cell lines. Therefore, preliminary examination did not allow us to correlate the death of MCF-7 cells to the action of ISPHs. Later, observation of highly decreased rate of MCF-7 cells proliferation ranging from 64.9±1.4% to 74.8±2.2% confirmed the high antiproliferative capacity of ISPHs at 10 μg/ml compared to low dose. Therefore, natural antioxidants with less antiproliferative potential can be used at higher concentrations due to low toxicological effects compared to synthetic peptides [10]. Consequently, breast cancer cells exhibited the lowest rate of viability which varied from 43.9±1.8% to 65.7±1.6% when treated with the highest dose of peptides (20 μg/ml). The protein hydrolysates displayed a significantly high cytotoxicity at high dosage (20 μg/ml) which inhibited the growth of cells by more than 50 % in case of FII and FIII fractions.

Moreover, FII and FIII fractions of Indian salmon with the highest antiproliferative activities had MWs of 1 to 3 kDa and 3 to 5 kDa, respectively. The MW of bioactive peptides in our study was greatly higher than tuna dark muscle peptides with MW of 390 to 1400 kDa [33] and the antiproliferative peptide with MW of 440.9 Da obtained from anchovy sauce that induced apoptosis in human U937 cells by

increased caspase-3 and caspase-9 activity [34]. In contrast, other studies on buckwheat found peptides with MW of 3.9 kDa having antiproliferative activity against MCF-7 breast cancer and many other cell lines [12]. Comparison of these results with our study indicates that the antiproliferative capacity of bioactive peptides is not correlated with their MWs.

Additionally, composition and sequence of amino acids in peptides play an important role in their antiproliferative potential [34]. It is believed that cationic peptides promote cytotoxicity of peptides by interacting with anionic components of the cell membrane [34]. Moreover, positively charged amino acids such as Arg and His are also proved to enhance the cytotoxic activity of peptides. Since replacement of non-positive amino acids by positively charged amino acids in bioactive peptides resulted in 1.2 to 1.09 fold enhanced antiproliferative activity [35]. It is remarkable that ISPHs showed a good proportion of Arg that accounted for 2.53% to 3.87% of total amino acids which might be one of the reasons for their high cytotoxicity (table 2).

Moreover, hydrophobicity is also considered as one of the major properties that affect the anticancer activity of peptides [34]. Therefore, the significant antiproliferative capacity of ISPHs could be attributed to high concentration of hydrophobic amino acids in them which comprised of 38.49% (FI), 40.49% (FII) and 39.08% (FIII) of total amino acids. As mentioned before, the highest measures of hydrophobic and positively charged amino acids had been observed in FII fraction with peptides of 1 to 3 kDa which could partially explain the greater anticancer activity of peptides in this fraction. However, a detailed study on the amino acid sequence of these peptides is further recommended. It has to be mentioned that, in this study ISPHs were prepared by following the *in vitro* gastrointestinal digestion model to confirm the possession of same beneficial effects when consumed by humans.

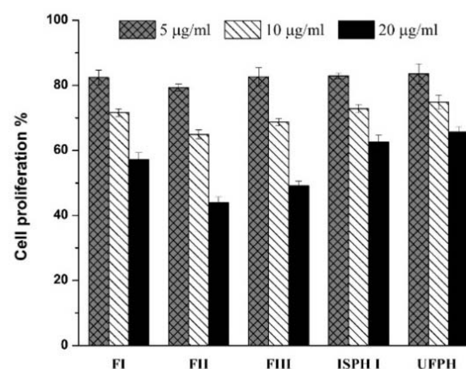


**Fig. 2: Effect of ISPHs and UFPH at different concentrations on cell proliferation of MCF-7 breast cancer cell line cultured on DMEM medium for 72 h. Each value is expressed as the mean±standard deviation (n = 3)**

**Amino acid composition of ISPHs**

The amino acid composition of ISPHs was determined in order to evaluate the influence of amino acid profile on antioxidant and antiproliferative activity of hydrolysates. It has to be noted that different parameters such as digestive enzymes used for hydrolysis, pH, temperature and enzyme/substrate ratio can affect the amino acid composition of protein hydrolysates [36]. The amino acid composition of FI (MW<1 kDa), FII (MW 1-3 kDa) and FIII (MW 3-5 kDa) are depicted in table (2). In relation to essential amino acids (EAA), FIII (50.56%) was found to be a rich source followed by FI (48.83%) and FII (48.77%) fractions.

**Table 2: Amino acid composition of Indian salmon protein hydrolysates**

| | FI | FII | FIII |
|---|---|---|---|
| Asp | 10.27 | 10.34 | 10.31 |
| Thr[a] | 6.18 | 6.02 | 6.45 |
| Ser | 4.79 | 4.14 | 5.09 |
| Glu | 16.14 | 16.37 | 16.33 |
| Gly | 3.11 | 2.83 | 2.65 |
| Ala* | 5.37 | 5.39 | 5.23 |
| Val*[a] | 5.44 | 6.18 | 6.12 |
| Met*[a] | 5.16 | 5.03 | 4.39 |
| Ile*[a] | 3.81 | 4.2 | 4.17 |
| Leu*[a] | 8.09 | 8.6 | 8.52 |
| Tyr* | 3.51 | 3.54 | 3.22 |
| Phe*[a] | 3.93 | 4.31 | 4.22 |
| His[a] | 3.47 | 3.55 | 3.3 |
| Lys[a] | 8.32 | 6.64 | 8.75 |
| Arg | 3.81 | 3.87 | 2.53 |
| Cys | 0.98 | 1.02 | 0.83 |
| Pro* | 3.18 | 3.24 | 3.21 |
| Trp[a] | 4.44 | 4.73 | 4.68 |
| HAA[c] | 38.49 | 40.49 | 39.08 |
| EAA[d] | 48.84 | 49.26 | 50.6 |

Values are expressed as±SD, n = 3., *Stars indicate hydrophobic amino acids., [a]Essential amino acids., [c]Total hydrophobic amino acids., [d]Total essential amino acids.

Three studied peptide fractions were dominated by Glu (15.69-16.33%), Asp (9.82-10.31%) and Lys (6.6-8.75%). Several amino acids such as Met, His and Cys are generally believed to have antioxidant activity. Accordingly, a good proportion of mentioned amino acids were observed in the studied peptides in which His accounted for 3.3% to 3.55%, Met varied from 4.39% to 5.16% and Cys contents ranged within 0.83% and 1.02% of total amino acids (table 2). Arg with guanidine side chain carrying positive charges plays an important role in the antioxidant activity of peptides. This critical amino acid was found constituting 2.53% (FIII) to 3.87% (FII) of total amino acids in ISPHs.

Moreover, HAAs are suggested to enhance the antioxidant activity of peptides and positively inhibit lipid peroxidation by increasing their solubility in lipids and facilitating a better interaction with free radicals. The total HAAs ranged from 38.49% to 40.49% with the highest level in FII peptides. As a result, good amount of Leu in ISPHs can significantly inhibit radical mediated lipid peroxidation.

Moreover, acidic (Asp and Glu) and basic (Arg and Lys) amino acids play an important role in quenching free radicals and promoting the antioxidant activity of peptides [37]. Asp and Glu had the highest amounts of 10.34% and 16.37% in FII fraction with the greatest

antioxidant and antiproliferative activity. Indeed, aromatic (Trp and Tyr) and nonpolar aliphatic amino acids are proved to possess free radical scavenging activity, and their presence in peptides helps inhibition of lipid peroxidation in living tissues [5].

## CONCLUSION

Studied ISPH fractions exhibited concentration dependent antioxidant and antiproliferative activities. The results revealed high antioxidant and free radical scavenging capacity of FII peptides in addition to their high antiproliferative activity against MCF-7 cells. The obtained peptides did not show any correlation of MW with their activities since the bioactive peptides (FII) in our study had the MWs of 1 to 3 kDa. However, hydrophobicity and presence of specific amino acids such as Arg, His, and Cys directly impacted the activity of peptides. In overall, ISPHs were identified as potent peptides with antioxidant and antiproliferative activities which could be used in food industries to confer functional and nutritional properties.

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## CONFLICTS OF INTERESTS

The authors declare no conflict of interest

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