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PURIFICATION AND IDENTIFICATION OF ANTIOXIDANT PEPTIDES FROM THE SKIN PROTEIN HYDROLYSATE OF MARINE FISH (*AURIGEQUULA FASCIATA)*

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ABSTRACT

Marine bio resources produce a great variety of specific and potent bioactive molecules including natural organic compounds such as fatty acids, polysaccharides, polyether, peptides, proteins, enzymes and lectins. Proteins from marine sources show as functional ingredients in foods because they possess numerous important and unique properties such as film and foaming capacity,[1] Bioactive peptides usually contain 3–20 amino acid residues and their activities are based on their amino acid composition and sequence. These peptides are reported to be involved in various biological functions such as anti hypertension, immune modulatory, antithrombotic, antioxidant, anticancer and antimicrobial activities, in addition to nutrient utilization. Peptides originating from various fish and shellfish have also exhibited good antioxidant activitie in different oxidative. Jellyfish collagen peptides ranging from have displayed high superoxide. In the current study, to evaluate the antioxidant active-ties of the peptides from freshwater clam, the enzymatic hydrolysates was prepared from the hot water extract of freshwater clam, and the corresponding antioxidant activities were evaluated varies methods.

KEYWORDS: Jelly fish, Collagen, hydrolysates, freshwater clam, shellfish.

INTRODUCTION

The holistic approach to link medicine and diet has ascertained that the fish. Because of the presence of specific biochemical ingredients in addition to their nutritive values, fish have a positive impact on an individual's health including his physical well-being and mental state. Fish also contains some health-promoting components beyond the traditional nutrients serve to prevent diseases. Fish is a low fat and/or good source of protein, essential for the healthy growth and maintenance of muscles and body tissues.[2] Fish offer scope for use as functional food and as sources of nutraceuticals. Great amount of marine fish species have been identified with potential nutraceutical and medicinal values. Consequently, a number of bioactive compounds have been identified including fish muscle proteins, peptides, collagen, gelatin, fish oil and fish bone. Some dietary proteins of fish cause specific effects going beyond nutrient supply. Bioactive peptides derived from various fish muscle proteins have shown various biological activities including antihypertensive, antibacterial, anticoagulant, anti-inflammatory, and antioxidant activities, and hence they may be a potential material for biomedical and food industries.[3]

Fish can serve as a source of functional materials, such as polyunsaturated fatty acids, polysaccharides, minerals and vitamins, antioxidants, enzymes and bioactive peptides. Fish is a fabulous food lots of variety in taste and texture, versatile and low in saturated fat. It's also low in calories-the perfect healthy diet food. Marine fish is a major source of high-quality protein, lipids, and a wide variety of vitamins and minerals.[4] These macromolecules and their derivatives show different pharmacological activities, which make the fish as a therapeutic diet. Modern technology has made it easy to explore the therapeutic importance of fish-based diet on cardiovascular diseases, neuro degenerative diseases, radicals-mediated diseases, and cancer. Among the complementary and alternative medicine.

(CAM) approaches used by Americans, natural products are the most popular. Recent interest in natural diets that can protect health has resulted in foods that have functionality from the nutritional point of view. A Joint WHO/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases met in Geneva from 28 January to 1 February 2002 has adopted a resolution regarding the primary and secondary prevention of chronic diseases and the reduction of their impact.[5]

India has a long coastal line of about 8000 km includes 13 main land states and 5 Union territories which comprises significant biodiversity. The east and west coast of India continues to be a large fishing ground in the south Asian region and is one of the largest marine product nations in the world. During the period of 2009, India's annual fish landings are about 3.16 million

tones.[6] Fish and its by-products represents as a rich source of nutrients of fundamental importance for health diets. It is not only rich in protein but also has a wide range of micronutrients including vitamins, minerals and polyunsaturated fatty acid.[7] Fish protein hydrolysates have been identified as the rich source of bioactive peptides with valuable pharmaceutical potentials.[8] Recent years, studies on bioactive peptides have received much attention in worldwide because of their health benefits. It has been shown that peptides made up to several amino acids can exhibit antioxidant activity, lower blood pressure and increasing immunity, antihypertensive, ant proliferative, anticoagulant, calcium-binding, antiobesity and antidiabetic activities.

Antioxidants from marine resources have attracted the attention of researchers as they are extracted from by-products of marine processing and do not have side effects. Fish protein hydrolysates from different species such as, yellow stripe trivially [9], yellow fin sole frame[10], herring[11], mackerel[12], muscles of *Nemipterus japonicus* and *Exocoetus volitans*[13], backbones of *Exocoetus volitans*[14], backbones of *Sphyraena barracuda* and *Lepturacanthus saval* [15] have been found to possess antioxidant activity. *Rastrelliger kanagurta* (Indian mackerel) is a pelagic shoaling fish, widely distributed in the tropical Indo-Pacific region and is an important revenue yielding variety in India. Despite the huge catch a larger amount is wasted and underutilized.[16] Reports have indicated that fish backbone is one of the major fractions of fish wastes, and it contains around 30% protein.[17] This protein could be good candidate as nutraceuticals..

Aurigequula fasciata

Aurigequula fasciata (Fig-1), the striped pony fish, is a species of pony fish native to the Indian Ocean and the western Pacific Ocean where it occurs in coastal marine and brackish waters. It is a large, robust, and deep-bodied pony fish. The dorsal and ventral profiles are equally convex. This species has a forward pointing mouth that opens strongly downward when protracted. The lower jaw profile is slightly concave. The mouth can extend about 15-20% of the SL. The greatest body depth is reached at the vertical from the dorsal-fin origin to the pelvic-fin origin. The dorsal- and pelvic-fin origins are located along the same vertical. The anal-fin origin is at a vertical with the first or second dorsal fin-ray [18]. The dorsal head profile is strongly concave with a large hump in the nuchal region. There is strong concavity dorsal to the orbit formed by this hump and the slightly convex dorsal profile in the region extends from the mouth to the area dorsal to the orbit. The teeth are small and villi form. The lateral line is complete and includes about 50 to 60 scales. The chest is asquamate; the entire triangular region between the margin of the opercle, the pectoral fin base, and the posterior margin of the pelvic fin is asquamate. The entire body is silvery. The head and asquamate chest region are silvery-white. The fin-spines are silvery. There is a light yellow tint to the membrane of all fins, particularly the membrane between the anal-fin rays.[19] There is little coloration in the caudal fin. Their dorsal flank has a pigmentation pattern of 10-15 broad yellow vertical lines that extend slightly ventral to the lateral line. Ventral to the lateral line and near the midline of the flank[20], the lines break into broad rounded dashes. The pectoral-fin axial and the base of the pectoral fin have a strong yellow coloration. The pelvic fins are white.

Fig. 1: *Aurigequula fasciata.*

MATERIALS AND METHODS

Collection and preparation of fish protein hydrolysates

Fresh *Aurigequula fasciata* was purchased from a local wet market in K.K.Nagar, Chennai, Tamil Nadu, and washed, eviscerated and hand filleted with the muscle and by-product (head, frames, and tail) separated. *Aurigequula fasciata* by products was then minced using a blender, packed in polyethylene bags, frozen, and stored at -20^{o}C until further use.

Enzymatic hydrolysis reaction

Minced *Aurigequula fasciata* was thawed overnight in a cold room (4^{o}C), then 15% w/v of minced TB was mixed with 50 ml of 50mM phosphate buffer solution (PBS, pH 7.5) and the mixture pre-incubated at 60^{o}C for 20 min prior to adding the 2.5% w/w alkalis' enzyme to initiate the enzymatic hydrolysis reaction. After 120 min of hydrolysis, the reaction was terminated by heating the mixture in a water bath at 90qC for 15 min with occasional agitation. The mixture was immediately cooled on ice, sediment at 10000 rpm for 20 min in a refrigerated high speed centrifuge and the supernatant was collected and the degree of hydrolysis (DH) was measured using OPA method [21]. Fish protein hydrolysates was then dialyzed and characterized.

Fish protein hydrolysates SDS-PAGE analysis

SDS-Poly acryl amide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of.[22]

Reagents
Stock solutions

Solution A	1.5 M Tris HCl buffer (pH 8.8) with 0.4 % (w/v) SDS
Solution B	0.5 M Tris HCl buffer (pH 6.8) with 0.4 % (w/v) SDS
Solution C	30 % (w/v) acryl amide with 0.8 % bis acrylamide
Solution D	1.4 % Ammonium per sulphate
Solution E	1 % SDS
Solution F	N,N,N,N' tetra methyl ethylene diamond (TEMED)

Preparation of gel

Separating gel [10 % (w/v)]		**Stacking gel [5 % (w/v)]**	
Solution A	0.75 ml	Solution B	0.38 ml
Solution C	2.0 ml	Solution C	0.5 ml
Solution D	0.3 ml	Solution D	0.15 ml
Solution E	0.6 ml	Solution E	0.3 ml
Distilled water	2.6 ml	Distilled water	1.98 ml
Solution F	0.005 ml	Solution F	0.005 ml

Tank buffer (pH 8.3)		**Sample Buffer**	
Tris	3.0 g	Glycerol	2.0 ml
Glycine	14.4 g	β-mercaptoethanol	1.0 ml
SDS	1.0 g	10 % SDS (w/v)	4.0 ml
Distilled water	1.0 L	Solution B	1.7 ml
		Bromophenol blue (aqueous)	0.2 ml
		Distilled water	0.6 ml

Procedure
The enzyme solution was mixed with an equal volume of sample buffer, boiled in a water bath for 3 min, cooled and added to the wells then the power supply was connected with cathode in the upper tank and anode in the lower tank. Electrophoresis was carried out at room temperature with constant voltage and 20 mA current supplies was maintained until the tracer dye reached 0.5 cm above the lower end.

Staining of separated proteins
At the end of electrophoresis, gel was removed and stained with silver staining method.[22] After staining, the gels were stored in 7 % (v/v) acetic acid.

Reducing power determination of fish protein hydrolysates
Fe (III) reduction is often used as an indicator of electron-donating activity, an important mechanism for phenolic antioxidant action.[23] The reducing power of the crude extracts was determined by spectrophotometric method of Yen and Chen (1995). The crude extracts (5-20 µl/ml) was mixed with 2.5 ml of 0.2M Potassium phosphate buffer (pH-6.6) and 2.5 ml of 15 Potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50°C for 20 minutes, then rapidly cooled, mixed with 2.5 ml of trichloroacetic acid and centrifuged at 5000 rpm for 3 minutes. An aliquot (2.5ml) of supernatant was diluted with distilled water (2.5ml) and 0.5 ml of 1% Ferric chloride was added and allowed to stand for 10 minutes. The absorbance was read spectrophotometrically at 700 nm. Increased absorbance indicates increased reducing power. Vitamin C was used as positive control.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay of fish protein hydrolysates
Radical scavenging activity using the ABTS method was performed according to the method described in an earlier study [24]. Different concentrations (equivalent to 5, 10, 15 and 20 µl/ml) of *Aurigequula fasciata* fish protein extracts, and BHA were taken in different test tubes. The volume was adjusted to 100 µL by adding methanol. Five milliliters of 0.1 m*m* methanol solution of ABTS was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27 °C for 20 min. The control was prepared as described previously without any extract, and methanol was used for the baseline correction. The changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\% \text{ radical scavenging activity} = \frac{(\text{control OD} - \text{sample OD}) \times 100}{\text{control OD}).}$$

Assay of nitric oxide-scavenging activity of fish protein hydrolysates
In this experiment, 1 ml of sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with varying concentrations of antioxidant peptides of *Aurigequula fasciata* (5-20 µg ml-1) dissolved in water and incubated at room temperature for 150 min. The

same reaction mixture, without the extract but with an equivalent volume of water, served as control. Following the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2 % H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read spectrophotometrically at 546 nm. Vitamin C was served as positive control.[25]

Antioxidant activity in a hemoglobin-induced linoleic acid system of fish protein hydrolysates

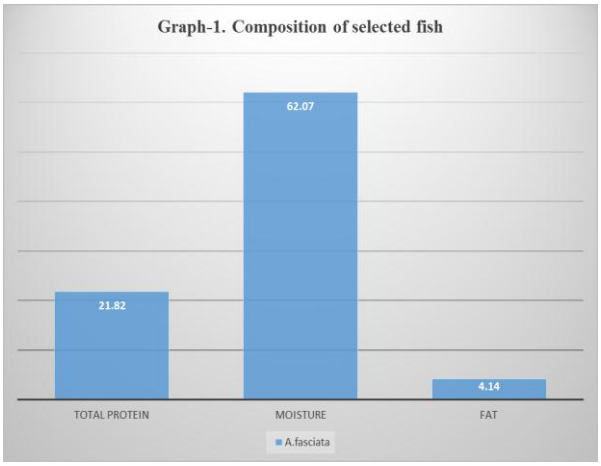
The antioxidant activity of *Aurigequula fasciata*peptide extract was determined by a modified photometry assay (Song-Hwan and Hyung-Joo, 2007). Reaction mixtures (200 ml) containing 10ml extracts (10–400 mg), 1mM/l of linoleic acid emulsion, 40mM/l of phosphate buffer (pH 6.5) and 0.0016% hemoglobin, were incubated at 37 ^{0}C for 45 min. After the incubation, 2.5 ml of 0.6% HCl in ethanol was added to stop the lipid per oxidation. The amount of peroxide value was measured in triplicate using the thio cyanate method by reading the absorbance at 480 nm after coloring with 100 ml of 0.02M/l of $FeCl_3$ and 50 ml of ammonium thiocyanate (30g/100ml). Vitamin C was used as positive control.

RESULTS AND DISCUSSION

Composition of selected fish

The chemical composition of food is very important not only from the perspective of human nutritional health, but also for assessment of the potential development and application of food materials in a food system. Proximate analyses were conducted on selected fish (Graph-1). Several studies have shown that the crude protein content of fish by-products varies from 8 to 35%, *A.fasciata* was within this range, indicating that it possessed considerable protein content.

The above graph shows the composition *A.fasciata* fish sample.



Purification of protein hydrolysates from *A.fasciata*

The cell free extract of *A.fasciata* was collected after extraction with 50mM phosphate buffer and its proteins were precipitated by salting out with ammonium Sulphate (70%). The crude protein preparation was dialyzed, concentrated by lyophilization and used for further analysis. The flow chart for the purification of protein hydroxyslate is presented in Fig-2 and Table-1.

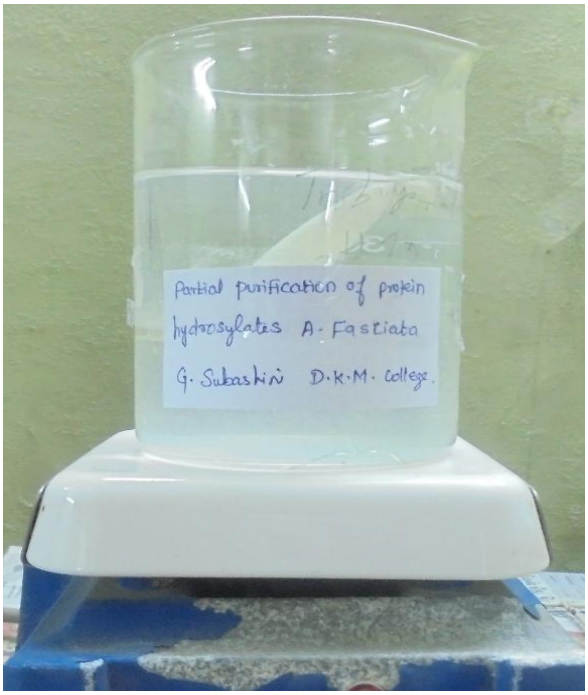


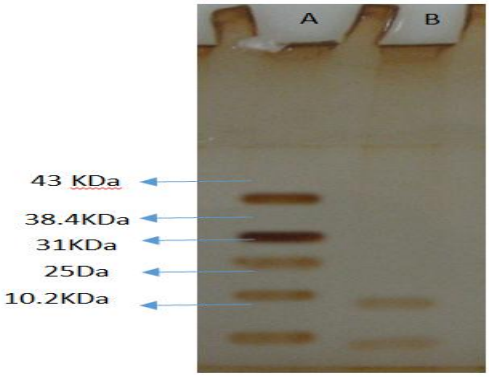
Fig. 2: Partial Purification of protein hydrolysates from *A.fasciata* by Dialysis.

Table 1: Purification yield of antioxidant peptides obtained from *A.fasciata*muscle.

Purification steps	Volume (ml)	Concentration of protein (mg/ml)	Total concentration of Protein (mg)	Total Hydroxyproline content (mg)	Specific activity	Purification fold	Yield (%)
Crude extract	100	40	4000	16800	4.2	1	100
Dialysis	80	30	2400	12400	5.1	1.2	73

Characterization of the molecular weight of *A.fasciata*

Characterization of the molecular weights of *A.fasciata* by SDS-PAGE showed the presence of strong bands ranging between 7.6 kDa, which indicated that *A.fasciata*protein hydrolysate was able to produce small-sized peptides in 120 min (Fig-3). Fish protein hydrolysates with high nutritional values should be rich in low molecular weight peptides, and the successful production of such desired peptides from TB indicated its potential application in functional food products.



A- Standard marker; B- dialyzed antioxidant peptides

Fig. 3: Characterization of the molecular weight of *A.fasciata.*

Reducing power determination of protein hydrolysates from *A.fasciata*

The reducing power of the protein hydrolysates from *A.fasciata* was determined. The protein hydrolysates reduced Fe^{3+} to Fe^{2+}. The formation of perls Prussian blue was measured at 700nm. The increasing in absorbance showed the increase in reduction. The increases in concentration of the protein hydrolysates from *A.fasciata* ranges from 5 to 20 µl/ml. The increase in concentration of the extract protein hydrolysates showed increase in reducing property. The maximum reducing property was found at 20 µl/ml protein hydrolysates (Fig-4 and Graph-2).

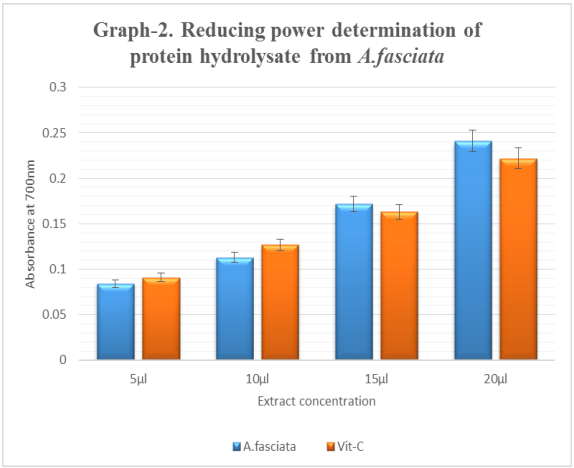


Fig. 4: Reducing power determination of protein hydrolysates from *A.fasciata.*

A- Control

B- 5 µl/ml of protein hydrolysate

C- 10 µl/ml of protein hydrolysate

D- 15 µl/ml of protein hydrolysate

E-20 µl/ml of protein hydrolysate

Scavenger effect of different concentrations of protein hydrolysates from *A.fasciata* expressed as capacity to bleach the stable ABTS radical

The free-radical scavenging activity of protein hydrolysates from *A.fasciata* was also tested by their ability to bleach the stable ABTS radical. This assay provided information on the reactivity of test compounds with a stable free radical. Because of its odd electron, ABTS gives a strong absorption band at 517 nm in visible spectroscopy (deep violet colour). As this electron becomes paired off in the presence of a free-radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up protein hydrolysates showed a significant and dose dependent ABTS quenching capacity, higher concentration of protein hydrolysates from *A.fasciata*20µg/ml was more efficient than lower concentration (Graph-3 and Fig-5).

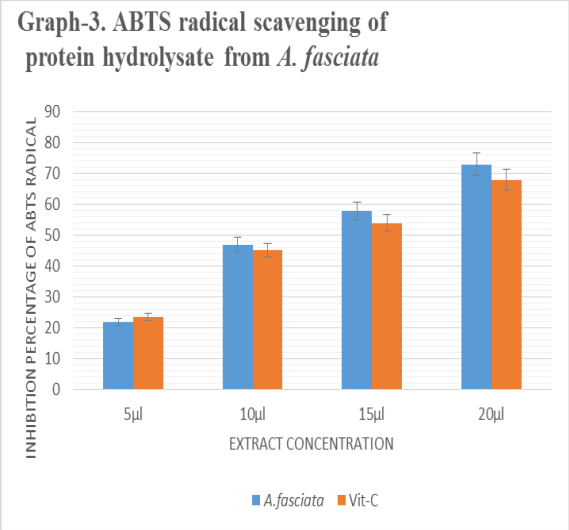
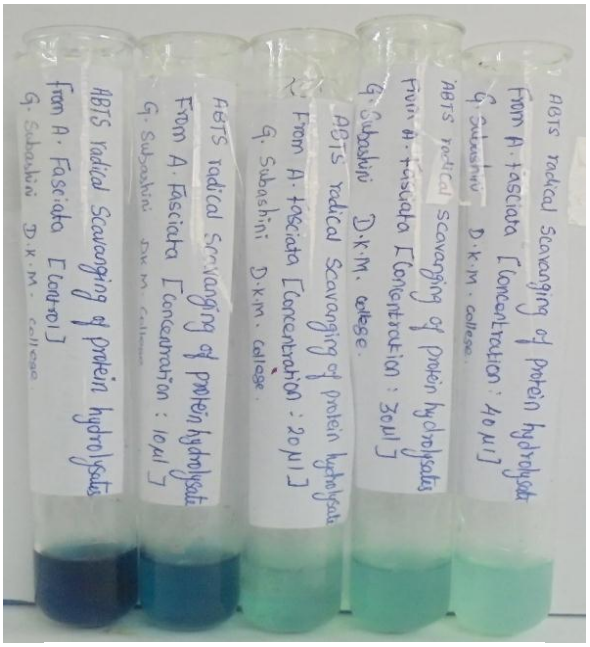


Fig 5: Scavenger effect of different concentrations of protein hydrolysates from *A.fasciata* expressed as capacity to bleach the stable ABTS radical.



A B C D E

A- Control
B- 5 µl/ml of protein hydrolysate
C- 10 µl/ml of protein hydrolysate
D- 15 µl/ml of protein hydrolysate
E-20 µl/ml of protein hydrolysate

Antioxidant activity in Hemoglobin induced linoleic acid of protein hydrolysates from *A.fasciata*

The antioxidant activity of protein hydrolysates from *A.fasciata*was determined by using the hemoglobin induced linoleic acid system. The antioxidants peptides extract act as electron donors and react with radical and stabilize the radical to terminate radical chain reaction. The maximum inhibitory activity was 81 % in 20µg/ml concentration of protein hydrolysates (Fig-6 and Graph-4).

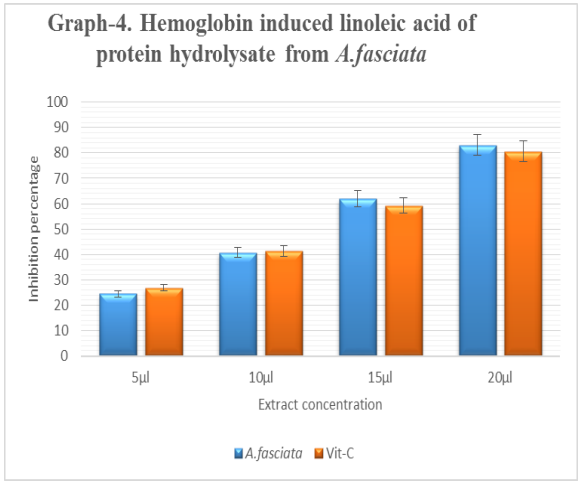
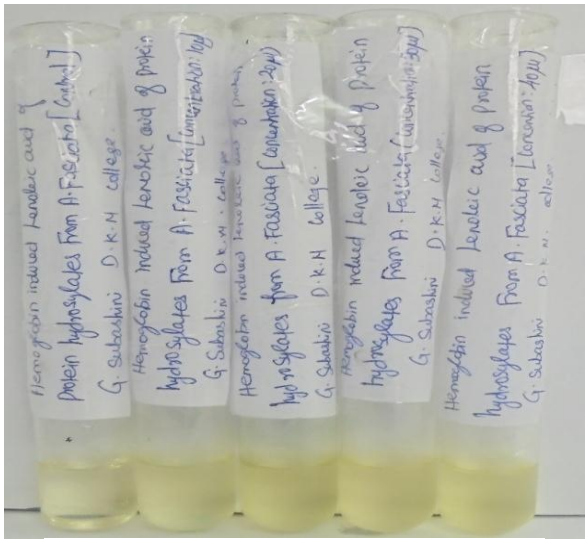


Fig 6: Antioxidant activity in Hemoglobin induced linoleic acid of protein hydrolysates from *A.fasciata.*



A B C D E

A- Control
B- 5 µl/ml of protein hydrolysate
C- 10 µl/ml of protein hydrolysate
D- 15 µl/ml of protein hydrolysate
E-20 µl/ml of protein hydrolysate

Nitric oxide-scavenging activity of protein hydrolysates from *A.fasciata*

The scavenging of Nitric oxide by the extracts was increased in dose dependent manner. Graph-5 illustrates a significant decrease in the Nitric oxide radical due to the scavenging ability of protein hydrolysates from *A.fasciata*. The protein hydrolysates showed maximum activity of 68.51% at 20µg /ml (Graph-5 and Fig-6).

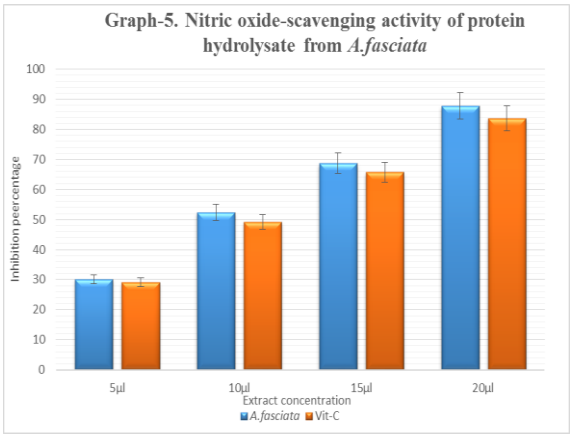
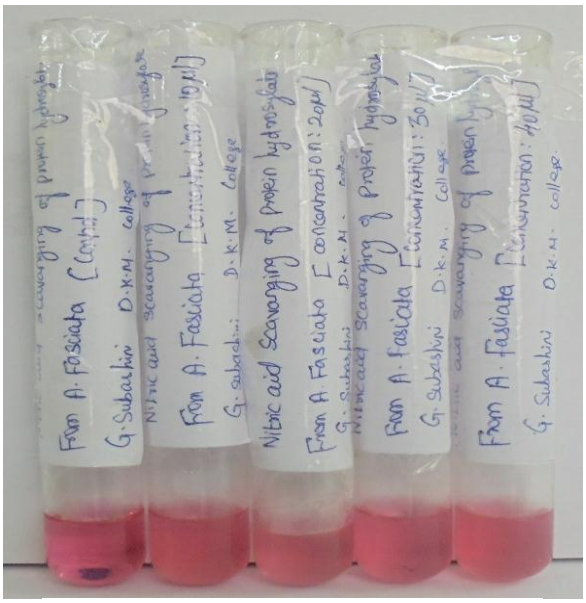


Fig 7: Nitric oxide-scavenging activity of protein hydrolysates from *A.fasciata.*



A B C D E

A- Control

B- 5 µl/ml of protein hydrolysate

C- 10 µl/ml of protein hydrolysate

D- 15 µl/ml of protein hydrolysate

E-20 µl/ml of protein hydrolysate

CONCLUSION

Antioxidants play roles in multiple reaction mechanisms. An efficient way of assessing antioxidants is to take into their account oxidation-reduction potentials, i.e., measuring their reduction powers. In the current study, the antioxidant activity of the protein hydrolysates from *A. fasciata*was evaluated via reducing power and the ABTS+•. radical. The reducing power of the hydrolysates was much stronger protein hydrolysates from *A. fasciata.* The enhanced antioxidant activity of the hydrolysates resulted from the opening and exposure of active amino acid residues, which were electron donors and reacted with free radicals to terminate the radical chain reaction. The TEAC value of the hydrolysates from *A. fasciata*was relatively lower than that from Pacific hake (*Merluccius productus*: 65.58 µg/mg). The antioxidant activity of a peptide depends not only on its molecular size, but also on its chemical properties, such as hydrophobicity and the electron transferring ability of its amino acid residues. Potassium ferricyanide is widely used to measure the reducing power of a hydrolysates fraction's antioxidant activity for which higher absorbance indicates higher reducing power. The reducing power for the 10.2with 25 kDa crude peptides was higher in absorbance than that Vitamin-C. The molecular weight of 10.2 and 25 kDa protein hydrolysates of *A.fasciata* plays a significant role in antioxidant activity, with lower molecular weight fractions possessing higher antioxidant activity. These activities include the chelation of ABTS, Hemoglobin induced linoleic acid and Nitric oxide scavenging, although Vitamin C (positive control) showed the highest antioxidant activity overall. However, this study established that the purified form of enzymatic hydrolysates of *A.fasciata* extracts have shown higher antioxidant ability.

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