Extraction of Novel Peptide With Ace Inhibiting Activity And Antioxidant Study From Anchovy's Fish [*Stolephours Indicus*]

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Abstract- *Due to advancement in technologies carbon footprint is increasing continuously due to continuous usage. designers are attracted towards energy efficiency in the internet of things(IOT) and this new area is named as green Iot. Lots of techniques have been used. Green automation is important in today's era. Study of different scrubbers is done to absorb carbon. after comparison efficient one is used for further processing.*

Keywords- Cloud Computing, Green Internet Of Things, Smart Phone, Android Application, Wireless Sensor Networks.

I. INTRODUCTION

The anchovy is a significant food source for almost every predatory fish in its environment, including the California halibut, rock fish, yellowtail, shark, chinook, and coho salmon. Protein hydrolysates from milk , soy and fishes contain peptides that possess angiotensin I converting enzyme [ACE] inhibitory properties and may help to prevent hypertension. Fish Consumption, Fish Oil, Omega-3 Fatty Acids are the drug which is used to treat hypertension. ACE inhibitors are major anti-hypertensive drugs[1]. An angiotensin-converting-enzyme inhibitor [ACE inhibitor] is a pharmaceutical drug used primarily for the treatment of hypertension [elevated blood pressure] and congestive heart failure [6]. Angiotensin I is converted to angiotensin II [AII] through removal of two C-terminal residues by the enzyme angiotensin-converting enzyme [ACE] [7]. ACE is a target of ACE inhibitor drugs, which decrease the rate of angiotensin II production. Hypertension [HTN or HT], also known as high blood pressure [HBP], is a long-term medical condition in which the blood pressure in the arteries is persistently elevated. High blood pressure usually does not cause symptoms. Angiotensin-I-converting enzyme [ACE] inhibitors like synthetic drugs are widely used to control hypertension. Antihypertensives are a class of drugs that are used to treat hypertension [high blood pressure]. Antihypertensive therapy seeks to prevent the complications of high blood pressure, such as stroke and myocardial infarction. Evidence suggests that reduction of the blood pressure by 5 mmHg can decrease the risk of stroke by 34%, of ischaemic heart disease by 21%, and reduce the likelihood of dementia, heart failure, and mortality from cardiovascular disease [3]. There are many classes of antihypertensives, which lower blood pressure by different means. Among the most important and most widely used drugs are thiazide diuretics, calcium channel blockers, ACE inhibitors, angiotensin II receptor antagonists [ARBs], and beta blockers [4]

II. MATERIALS AND METHOD

Collection of fish

The *Anchovies* fish (*Stolephorus indicus*) were collected from Ukkadam fish market (without any physical damage) Coimbatore , Tamil Nadu , India.

Preparation of muscle extract

Fish was dissected aseptically, the intestine was removed and it is cut into two parts of head muscle and tail muscle it was ground with phosphate buffer [pH 7.2] and the extract was collected and incubated for 24 hours. After incubation, the extract was centrifuged at 5000rpm for 5 min. The supernatant was collected and used for further studies.

Estimation of protein by lowry's method

Lowry et al method were used to analysis of protein content µg/g of protein was calculated by using standard graph of BSA.

Antioxidant activity

Dpph free radical scavenging activity

To the 0.5 ml of hydrolysed head and tail sample, added a 0.1 ml of methanol dissolved in DPPH solution to both a test tubes, mixed well and then 0.4 ml of 50 mM tris hydrochloric acid was added. Incubated the tubes at dark for 30 minutes and the optical density was measured at 517 nm using spectrophotometer. The free radical scavenging activity was calculated using ascorbic acid as standard.

Total antioxidant activity

Phospho molybdenum method was used to analysis the total antioxidant content. 1 ml of head and tail extract was mixed with 1 ml of reaction mixture [0.6 M Sulphuric acid, 28 mM Sodium phosphate, 4mM Ammonium molybdate] and incubated at 50˚c for 90 minutes. After heating the optical density was measured at 695 nm under spectrophotometer. The mg/g of total antioxidant was calculated using ascorbic acid as standard.

Preparation of hydrolysate

50 gram of fish extract was blended with 50 ml of distilled water, smashed and heated the sample with boiling water bath at 90˚c for 15 to 20 minutes and the sample was taken from the water bath blended the sample with mortar and pestle for 2 minutes, 5 ml of protease enzyme was added and heated at 55˚c for 1 hour in a boiling water bath. After heating, the sample was taken and added 5 ml of 1 M sodium hydroxide, again heated the extract at 80˚c for 15 minutes and centrifuged the sample to collect the supernatant of angiotensin enzyme and stored the hydrolysed enzyme at -20˚c for overnight. After over night incubation, the sample was used to calculate the % of Hydrolysate.

$$\% \text{ OF HYDROLYSATE} = \frac{B \times Nb \times 100}{\alpha \times M.P \times \text{mass weight}}$$

ACE inhibitor Study

The hydrolysate was dialysed using 75kDA membrane against phosphate buffer [7.2] solution for overnight. After that the filtrate was collected and the reading was measured at 242 nm and 284 nm under spectrophotometer along with the blank. The dialysed sample were used for ACE inhibitor study by adding 20 mM tri's hydrochloric acid and ACE substrate the mixture was incubated at 37˚c for 45 minutes and added 10 µl of ACE inhibitor, again the sample was incubated at 37˚c for 20 minutes. After incubation measured the optical density at 370 nm.

Extraction of bioactive peptides

Thin layer chromatography

TLC – [Thin Layer Chromatography plate 3X3Cm Merck Germany plate]

A silica gel coated TLC plate [3X3 cm Merck Germany plate] was used, and a thin line was drawn 2 cm from the bottom. On the bottom line, evenly spaced markings were made for spotting the amino acid standards, and for the sample. All the amino acid standards were prepared in1 ml test tubes in distilled water [concentration of 1mg/ml]. The prepared amino acids standards were spotted on the markings using capillary tubes. The spotting of amino acids was carefully done, in such a way that the spots were small [bigger spots might lead to an imprecise result]. After spotting, the plate was allowed to air dry. In the TLC tank, the solvent system consisting of butanol, glacial acetic acid and water in the ratio of 8:2:10 [the mobile phase] was prepared. The solvent system in the tank was exactly 1 cm from bottom of the tank. The TLC plate was then placed in the tank and was closed tightly. The solvent was allowed to run up to the top marked line on the plate. After that the TLC plate was carefully taken out, the line on the top of the sheet was the solvent front. The plate was allowed to completely dry, and then taken to the fume hood to spray 2% Ninhydrin in ethanol solution on it.

It was then kept in a hot air oven to dry at 50ºC for 5 min. The Retardation factor [Rf]value of each amino acid was calculated using the following formula

$$Rf = \frac{\text{Distance travelled by component}}{\text{Distance travelled by the solvent}}$$

Purification by gel filtration chromatography

Samples that exhibited the highest activity were fractionated by gel filtration chromatography using A SephadexG-25 gel filtration column [Hi Media][3 cm X 30 cm] was equilibrated with a phosphate buffer [pH 7.0]. The sample [5ml] dissolved in 6 ml of a 50 mM sodium phosphate buffer [pH 7.0], was injected onto the column and eluted with the same buffer for 2 hours at a constant flow rate of 1 ml/5min [5 fractions].

SDS PAGE

SDS-PAGE [sodium dodecyl sulphate-polyacrylamide gel electrophoresis] ,separates proteins

according to their molecular weight, based on their differential rates of migration through a sieving matrix [a gel] under the influence of an applied electrical field. Prepare the sample solution to be loaded into the wells by adding the required volume of sample buffer [4 parts of sample and 1 part of sample buffer]. The sample was incubated at 90ºC in a boiling water bath for 5 minutes. When the polymerization of the stacking gel gets over, remove the comb and the lower spacer strip carefully. Remove excess vacuum grease from the bottom of the gel by wiping with a piece of tissue paper. Remove the comb carefully and clean the wells using filter paper. Fill the lower reservoir and upper reservoir of the electrophoresis apparatus with the required volume of tank buffer .Fix the gel plate to the electrophoresis tank carefully with appropriate clips and clamps. Load the protein samples in the wells and equalize with the level electrophoresis buffer in the upper chamber by filling with electrophoresis buffer. Raise the level of buffer in the upper reservoir. Connect the electrodes to the power pack and the switch on the current and observe for the formation of bubbles which indicates the passage of current. Keep 10-15 mA /50-60 V for stacking gel and 15-20mA/75-100V for separating gel. Turn off the power supply when the tracking dye reached the bottom of the gel and transfer the gel to the staining solution for 1-2 hours. Later transfer the gel to destaining solution until the clear bands got visible.

Antimicrobial activity

For the antimicrobial study, Cultures of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were used for antibacterial activity and *Aspergillus niger*, were used for antifungal study.

Antibacterial activity- well diffusion method

The 24hrs old cultures of the pathogens were used in the study. Mueller Hinton Agar was prepared and sterilized at 121^0C for 15minutes. The antibacterial assay was carried out by the agar well-diffusion method .

Well diffusion method: Mueller Hinton agar plates were prepared and after solidification, 60 µl of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoneae* were poured and spread with sterile cotton swab and kept for drying for 2-3 minutes. Wells were made with cork borer in the diameter of 5mm and added distilled water as a negative control, 50µl of extract in separate wells. Erythromycin antibiotic disc was used as a positive control. All the plates were incubated at 37^0c for 24 hours. The diameter of zone of inhibition produced by the extract was measured in mm after the incubation period.

Antifungal activity

Standard fungal cultures of *Aspergillus niger*, were used. The fungal stock cultures were maintained on potato dextrose agar. Malt agar was made and swaped with the fungal culture. Three wells were made with cork borer in the diameter of 5mm and added 50 µl distilled water, hydrolysed portion of head and tail are poured into separate wells. The fungal plates were incubated at 29^0c for 3-5days. The diameter of zone of inhibition produced by the extract was measured in mm after the incubation period.

Anticancer activity

In the 96 well plate marked as a blank, medium, fish head portion, fish tail portion and control at 100 µl of DMSO were added to both blank and medium wells. Then fresh medium was added well and added a hydrolysed sample [25 and 50 µl] of fish head portion and tail portion to the respective wells..A cancer cell of He La was added to 5^{th} well which serve as control. It was incubated for 24 hours in a CO2 incubator. After incubation added 150 µl of DMSO, washed and then added a 10 µl of MTT DYE and incubated for 24 hours in a 5%CO2 incubator , The OD was read using ELISA reader [After measuring the reading] the % of cell death were calculated by following formula.

$$\text{Percentage of cell death} = \frac{\text{control absorbance reading} - \text{absorbance of treated}}{\text{control absorbance reading}} \times 100.$$

III. RESULT AND DISCUSSION

Estimation of protein by lowry's method

The amount of protein present in the head and tail muscle was found to be 152 µg/ml and 102 µg/ml.

Defatting of egg yolk with hexane and ethanol resulted in a white powder. The lipid was removed successfully resulting in a high percentage of protein, as determined by the Kjeldahl method. Protein content of the defatted egg yolk sample was 91.0% -0.2% compared with native egg yolk 30.2% _ 0.2.% [5].

DPPH free radical scavenging activity

The DPPH activity of enzyme present in the head and tail muscle was found to be 24 µg/ml and 14 µg/ml. The total

antioxidant of samples present in the head and tail muscle was found to be 96 µg/ml and 34 µg/ml.

The radical scavenging activity of carp roe protein hydrolysates [CRPHs] was assessed by using two in vitro free radical models, namely, DPPH and $ABTS^{+}$. When DPPH and ABTS radicals encounter a proton-donating substance such as an antioxidant, the radical is scavenged, and the absorbance is reduced by changing the color [Liu et al. 2010]. carp roe protein hydrolysates showed good DPPH and $ABTS^{+}$ radical-inhibiting activity in a dose-dependent manner. Intarasirisawat et al. [2012] and Chalamaiah et al. [2013]who reported that the DPPH and ABTS scavenging activity increased with increasing concentrations [2]

Acetyl choline esterase assay of protein hydrolysate

After dialysis of the extract, the protein activity was studied and it was found to be 20µg/ml and 5 µg/ml for head and tail respectively.

The acetyl choline esterase activity ,was hydrolysed ,dialysed and the anti inhibitory activity was found to be 19 µg/ml .

Purification by gel filtration chromatography

The enzyme used in the technique of gel filtration produce a 5 fraction.

The first fraction has 0.061 , second fraction has 0.093 , third fraction has 0.105 , fourth fraction has 0.149 and the fifth fraction has 0.126.

Extraction of bioactive peptides from fish sample

The amino acids present in the standard were found to be L . tryptophan , L . tyrosine and L . arginine. The amino acids present in the head sample were found to be L .tryptophan , L . leucine , L . valine , L . tyrosine , L . arginine. The amino acids present in the tail sample were found to be L .tryptophan , L . methionine , L . valine , L . tyrosine .

Fig 1 THIN LAYER CHROM ATOGRAPHY

SDS PAGE was done to determine molecular weight

Characterization of the molecular weights of head digested sample by SDS-PAGE showed the presence of strong bands ranging between 5.6, 29 and 32 k Da, which indicated that enzyme was able to produce small-sized peptides .

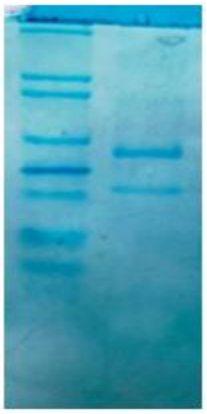

Fig 2 SDS PAGE

Antimicrobial activity

The antimicrobial activity was carried out by Agar well diffusion method .Antibacterial activity was carried out against four bacterial strains and maximum activity was observed against *E.coli* and Antifugal activity against *Aspergillus niger*.

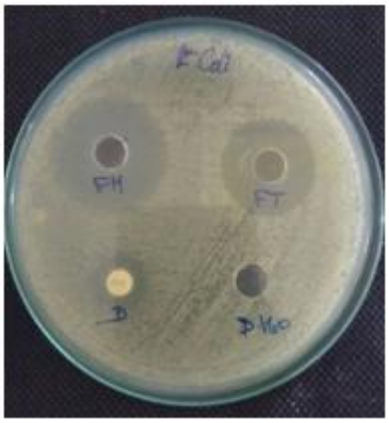

Fig 3 Antibacterial activity in *Escherichia coli*

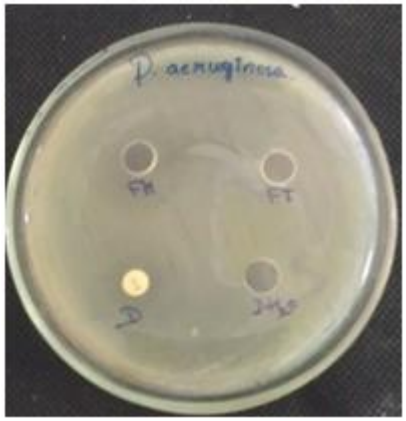

Fig 4 Antibacterial activity in

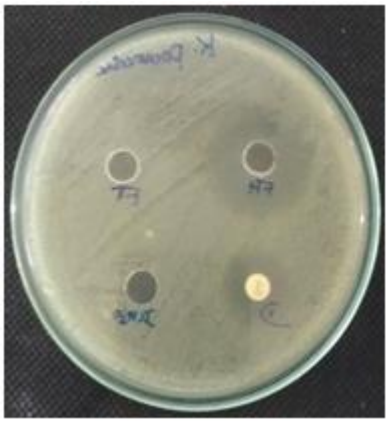

Fig 5 Antibacterial activity in
Pseudomonas aeruginosa
Klebsiella pneumoneae

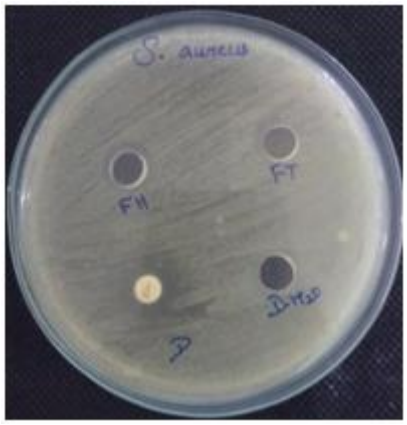

Fig 6 Antibacterial activity in Staphylococcus aureus

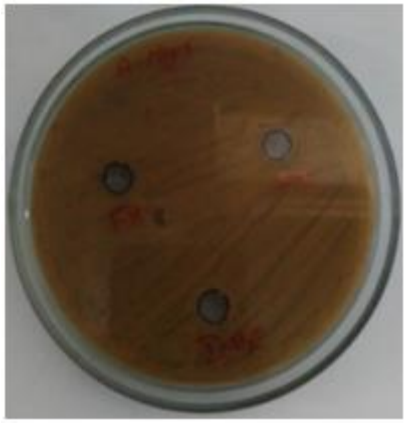

Fig 7 Antifungal activity in
Aspergillus niger

IV. ANTIMICROBIAL ACTIVITY

Effect of anticancer activity:

The % of cell death were calculated by following formula.

$$\text{Percentage of cell death} = \frac{\text{control absorbance reading} - \text{absorbance of treated}}{\text{control absorbance reading}} \times 100.$$

The percentage of cell death of 25 µl fish head and tail was 12.47 % and 10.23 % of cell death. The Percentage of cell death of 50 µl fish head and tail was 27.38 % and 25.03 % of cell death.

Control

Treated

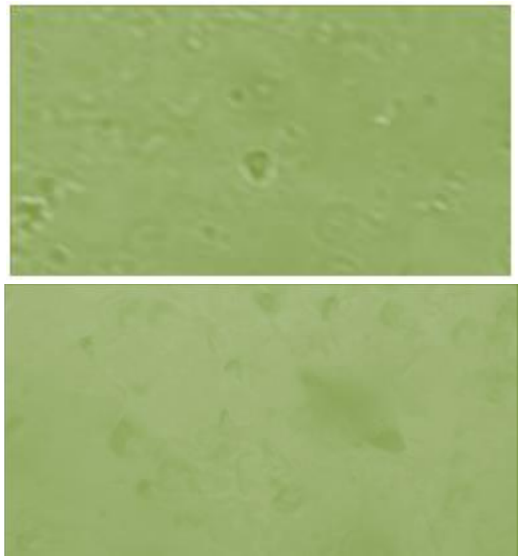

Fig 8 Anticancer activity

V. SUMMARY AND CONCLUSION

In recent years, increased attention has been paid to the biological activity of peptides of marine origin.. Human gastrointestinal enzymes generated peptides with weaker ACE inhibitory activity of the hydrolysates than the activity of peptides from hydrolysates obtained with commercial enzymes.

In the present study, the acetyl choline esterase inhibitor study was carried out and we have identified a new peptide from Anchovy's Fish (*Stolephours indicus*) exhibiting potent inhibition of ACE, which herald a fascinating opportunity as neutraceutical or therapeutic application. The excellent results of this study shed insights, into the potential of new bioactive peptide as therapeutic alternatives in the treatment of hypertension as they additionally exert antioxidant and antimicrobial activities.

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