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Anti-oxidant, Anti-inflammatory and Anti-Atherosclerotic Activity of Bioactive Peptide HPAEDR Isolated from *Catla catal* Muscle on LPS Induced Inflammation on 246.7RAW Macrophage Cells and HCF Induced Hyperlipidemic Zebrafish Larvae

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Abstract

A muscle wasdissected from the *Catla catla*fish and enzyme hydrolysis was done using various digestive enzymes such as pepsisn, protease, papine, trypsin and alcalase at verity of time intervals (0^{th}, 3^{rd}, 6^{th}, 9th and 12^{th}) hour respectively. Followed by, the amino acid composition was identified and the confirmative assays such as, the anti-oxidant assays (DPPH and Hydroxy radical scavenging activity) and anti-inflammatory assays (HRBC and AD) were done for various peptide hydrolysate. The active hr was identified as 9^{th} hr alcalase hydrolysate which was purified through Ultrafiltration (>30 kDa, 30-10 kDa, 10-3 kDa and <3 kDa). These fractions were again studied for its anti-oxidant and anti-inflammatory activity. Based on the results obtained, the active fraction was identified as 10-3kDa which was further purified and identified using Gel filtration chromatography and LC-MS/MS as HPAEDR (723.76 Da). Further, for *in vitro* and *in vivo* studies the peptide derived from CCM was synthetically designed with 98% purity (PhtdPeptides Co., Ltd. Zhemgzhou, China). Additionally, the physiochemical properties (Solubility, emulsifying properties and foaming properties) of these fraction was studied. Finally, the purified fraction was tested for *in vitro* activity through cell viability, COX-2 production, NO production and TNF-α production. Moreover, the *in vivo* protective effect is tested on Zebrafish larvae. The results suggest that the active purified peptide fraction isolated from Catla catla muscle has a strong natural anti-oxidant, anti-inflammatory and cholesterol reduction activity which can be used in functional foods and pharmaceuticals.

Keywords: anti-inflammatory; antioxidant; peptide; Zebrafish larvae; 246.7RAW macrophage; inflammation.

Abbreviations: HCF-High cholesterol food; AD-Albumin denaturation assay; HRBC- Human red blood cell membrane stabilization assay; CCM-*Catla catla* mussel

INTRODUCTION

Generally, fishes are considered as easily putrescible food hence, it starts to blemish as soon as it was harvested. To overcome these problems, processing and packaging methods such as drying and smoked fish that are required to increase its shelf life (Majidiyan et al. 2022). Moreover, the fish waste such as bones, muscles and viscera are not disposed in a proper way (Hotel 2001). The fish muscles are rich in proteins and collagen which can be used in development of novel pharmaceutical and nutritious functional food (Hadidi et al. 2021; Zamorano-Apodaca et al. 2020).

These collagen and muscle proteins are rich in amino acids such as glycine and proline which can be hydrolyzed using digestive enzymes to develop a bioactive peptide (Nurilmala et al. 2020). These enzymatic production of bioactive peptides will have various productive activities such as, anti-inflammatory, anti-oxidant and anti-microbial activities (Qian et al. 2020). The peptide isolation via enzyme hydrolysis is a productive and non-toxic method because even when it comes to provide non-synthetic hydrolysed peptide it will be less toxic than chemical or solvent based extraction. Mostly, digestive enzymes such as trypsin, pepsin, papine, protamex and alclase are used in enzyme hydrolysis, where alclase is considered as one of the active enzyme with ability to create bioactive peptide especially from fish species (Qiu et al. 2019). Salmon skin (Grundtman 2012) and Yellow tuna (Ray et al. 2019) are some of the examples from which bioactive peptide is isolated using alclase enzyme.

The reactive oxygen species (ROS) is one of the major markers which caused verity of oxidative stress related diseases such as, cardiovascular diseases and cancer (Huertas-alonso et al. 2021). Fish isolated bioactive peptide are a good source of anti-oxidant molecules which can reduce ROS production. Moreover,

the research shows that the peptides with anti-oxidant activity will posses other important activities such as anti-inflammatory and anti-microbial activities (Taheri et al. 2014). Carpfish (Catla catla) is one of the common fish that lives on fresh water (Zamora-Sillero et al. 2018). Fresh water fish production highly depends on carp fish because it is available globally (Franěk et al. 2021). Many studies show that fishes can posses a high anti-oxidant activity when enzyme hydrolysis is done (Chalamaiah et al. 2015). In this current study, we had investigated the anti-inflammatory and anti-oxidant activity of the bioactive peptide isolated from the carp fish. Additionally, we have investigated the gene expression and toxicity studies on macrophage cells and Zebrafish larvae.

MATERIALS AND METHODS

Sample Collection

The *Catla catla* was obtained from coastal area Thoothukudi (8° 45' 50.9976" N 78° 8' 5.4024" E) of Tamil Nadu, India and was verified from Zoological Surveyof India (ZSI), Chennai.

Chemicals Used

The chemicals used in this study were as follows: OPA, DTT, DPPH, pepsin, trypsin, papain, protamex, alcalse Sephadex G-25, LPS, phosphatase inhibitor (Sigma). Other chemicals were purchased commercially

Proximate Analysis and Amino Acid Quantification

The Catla catla muscle (CCM) dissected and the protein percentage is measured (Covey et al., 1991). The other parameters of the proximate analysis were also measured using commercially available kits. The CCM was hydrolyzed using HCL at 100 degree celcius for whole day and sent for HPLC to quantify the percentage of essential and non-essential amino acids present (Waterborg and Matthews 1984).

Enzyme Hydrolysis

The CCM was hydrolyzed using various digestive enzymes such as, pepsin, trypsin, papin, protamex and alcalase at respective pH and temperature (Adler-Nissen 1979). The extraction buffer was used to hydrolyze the CCM. The samples were taken for lyophilisation at different time intervals 0^{th} hr, 3^{rd} hr, 6^{th} hr, 9^{th} hr and 12^{th} hr respectively. Finally, the various lyophilized samples were used to determine the anti-inflammatory and anti-oxidant activity of the peptide hydrolysate.

Acid Hydrolysis

The Degree of hydrolysis percentage (DH %) was calculate using OPA method where, the various peptide hydrolysate were taken and dissolved in 1mg/ml concentration and 400 µL of sample was mixed with 300 µL of OPA after 20 minutes incubation at 37 degree Celsius the OD was measured at 340nm (Chandra et al. 2012).

Determination of Anti-Oxidant Activity

For DPPH assay, 50 µL of each peptide hydrolysate (1mg/ml) was mixed with 500 µL of DPPH (95% 1:1 v/v ethanol) and kept in 37 degree celcius for 30 minutes and the OD was measured at 517nm. The ascorbic acid was used as a positive control (Haghani et al. 2021).

For hydroxyl radical scavenging assay, 200 µL of each peptide hydrolysate (1mg/ml) was mixed with 900 µL of PBS, 100 µL of EDTA, 100 µL of ferrous sulphate and 500 µL of deoxyribose followed by, 250 µL of H202 and incubated for 30 minutes at room temperature. Finally, the OD reading was taken at 532 nm. The ascorbic acid was used as a positive control (Ghanbari et al. 2016).

Determination of Anti-Inflammatory Activity

In HRBC assay, the blood sample was collected from volunteries and mixed with equal volume of alsever solution and centrifuged at 1000 RPM for 15 min followed by, the addition of isosaline and incubated for 30 minutes at room temperature and finally, the absorbance is measured at 560 nm. The diclofenac was used as a positive control (Mizushima 1966).

In albumin denaturation (AD) assay, the sample is mixed with specimen 1% BSA and the mixture is incubated for 30 minutes at room temperature additionally, kept in water bath (60 degree Celsius) for 5-6 minutes and the OD reading was measured at 340nm. The diclofenac is used as a positive control (Rajapakse et al. 2005).

Separation, Purification and Identification of Peptide Sequence

Ultracentrifugation was done for the active hour (9^{th}) hydrolysate with different cutoff units (3, 10 and 30 kDA) to separate peptide hydrolysate into various fractions based on molecular weight. Then gel filtration coromatography was performed to purify the active fraction by maintain 1000 µL per minute speed at 280nm. The obtained fractions were again tested for inhibition activity and protein percentage (Ranathungaet al., 2006). Finally, the amino acid sequence of the purified fraction was identified using LC-MS/MS as HPAEDR (**723.76 Da**) and the results obtained were examined using Bio Tools Software.

Physiochemical Properties of Peptide

To determine the solubility of peptide the peptide was mixed with 1ml of water with a pH of around 3-9 and the mixture was centrifuged (8000 RPM) for 20 minutes. Additionally, to determine the protein content, sodium hydroxide was used as solvent (Ranathunga et al., 2006).

For emulsifying property, 1ml of maize oil was mixed with 30ml of peptide solution followed by

adjusting the pH between 2-10 respectively and the mixture was centrifuged (20,000) for 1 minutes. Then 0.5ml of SDS was added and the OD was measured at 500nm after 10 minutes incubation (Coveyet al.,1991).

To examine the foaming property the pure peptide was taken (200 µL) and centrifuge at 16000 rpm for 1 minutes. Finally the entire volume of the solution was determined (Pearce and Kinsella 1978).

In Vitro Assessment Peptide Activity on 246.7RAW Macrophage cells

RAW264.7 macrophage cells were cultured in DMEM media along with 10 percent FBS and 1 percent (v/v) antibiotic at 36 °C in a 5 percent CO2 incubator.

Cell Viability

RAW264.7 macrophage cells (1 104 cells/well) are incubated for 3–4 h at 37 °C with 50–1000 g/mL of pure peptide concentrations. After 1X PBS washing, 20 L of MTT reagent was added afterwards. After 3 hours of incubation, 200 L of DMSO was added to each well, and the OD was measured at 570 nm. Triton X-100 served as control substance (Mosmann 1983).

Determination of Nitrous Oxide Production

RAW264.7 macrophage cells (1 104 cells/well) were incubated for one hr at 37 °C with doses ranging from 60 to 260 g/ml, followed by a 24-hour incubation at 37°C with LPS (1 g/mL). Later, Griess reagent (1:1) was added to the supernatant and maintained for 10 minutes. Finally, OD was measured at 540 nm was observed (Nambiar et al., 2015).

Determination of Pro-Inflammatory Cytokine Synthesis

The cells (1 104 cells/well) were treated with peptides (50–250 g/mL) and LPS (1 g/mL) at 37 °C and 5% CO2 for one day; Diclofenac served as the reference. The quantitative analysis was performed by measuring the absorbance at 450 nm. In addition, standard concentrations versus absorbance values were used to produce standard curves.

In vivo Activity of Purified CCM Peptide on Zebrafish Larvae

Zebrafish maintenance and feeding

Zebrafish were purchased from local shops and grown and maintained in a cycling zebrafish aquaculture system at 28 °C with a 14:10 light-dark cycle. Factors such as pH, ammonia and nitrite concentrations are maintained to ensure water purity. A cholesterol diet (HCD) for zebrafish was prepared from a cholesterol solution in diethyl ether (Sigma) to obtain a cholesterol content of 4% (w/w) in artificial brine shrimp after evaporation of the ether. Five-week-old zebrafish were fed a diet three times a day. The control group was given regular fish kernels and the experimental group was given purified CCM peptide.

Cholesterol Measurement

The control and experimental Zebrafish larvae were euthanized using cold PBS and homogenized. By centrifugation at 1500 g at 4° C. for 5 minutes the supernatant was separated from 10 zebrafish larvae in each group. Total cholesterol was measured using a commercially available test kit.

In vivo Microscopy

For in vivo microscopy, sedated fish larvae were stored in droplets containing tricaine in a sealed, temperature-controlled environment. Zebrafish larvae were observed under a microscope. Pictures of zebrafish larvae were collected every 200 nm and all images were inspected and processed using software.

Gene Expression and Survival Percentage

The expression of TNF-α and NF-Kβ is studied on Zebrafish larvae and the survival percentage was calculated during the feeding and peptide treatment

Statistical Analysis

All the experiments were done in triplicate and the results were represented in the mean ± SD. The analysis was done using Graphpad Prism 8.

RESULTS AND DISCUSSION

Proximate Analysis of CCM

The muscle protein percentage was determined to be 33.67 ± 1.68% which has a similarity with other protein percentage (Gökoolu and Yerlikaya 2003). The percentage of other parameters of proximate analysis is shown **(Table 1)**. Other studies reveals that higher protein percentage will eventivually have higher possibility of getting active bioactive peptide.

Table 1. Protein percentage of BAF.

Proximate analysis	Percentage (%)
Protein	35.77 ± 0.46
Moisture	54.53 ± 0.03

(Table 1) - Protein percentage and moisture of BAF (mean ± SD)

Amino Acid Composition of CCM

The anti-oxidant, anti-inflammatory and physiochemical properties of peptides are related to the amino acid composition of the samples (Chen et al. 2017). The amino acid composition of the CCM is shown in the **table 2**. The results shows that, it has both essential and non-essential amino acids where it has higher percentage of amino acids such as, Histidine and glutamine which is highly known amino acids for its anti-oxidant and anti-inflammatory property (Liu et al. 2010). It also has

arginine and lysine amino acids which are responsible for high anti-microbial activity (Chi et al. 2014; Liu et al. 2010). Observed amino acids are known to posse's anti-oxidant activity of peptides (Alemán et al. 2011).

Table 2. Amino acid composition of CCM.

Amino acids	***CCM* (%)**
Asparticacid	15.7
Glutamic acid	25.7
Serine	19.2
Histidine*	7
Glycine	0.9
Threonine*	3.0
Arginine	17.3
Alanine	4.2
Tyrosine	6.9
Methionine*	0.9
Valine*	0.9
Phenylalanine*	5.2
Isoleucine*	6.0
Leucine*	9.5

*Essential amino acids

(Table 2) - It shows the amino acids composition present in the acid hydrolysis sample of *Oliva oliva* visceral mass

Percentage of Protein Hydrolyzed (DH %)

The percentage increases in the 9th hr hydrolysate as, 99.69 ± 1.79 %, 89.7 ± 1.54 %, 66.89 ± 0.85 %, 65.99 ± 0.77 % and 60.42 ± 0.39 % for trypsin, alcalase, papine, pepsin and protamex hydrolysate respectively **(Figure 1).** Similar studies show that the DH % can be calculate in the same method for marine organisms (Ray et al. 2019). Other studies shows that the pepsin and alclase has more DH% that other enzymes. In this study, we have used OPA method of DH % estimation which is easy to perform and cheaply affordable.

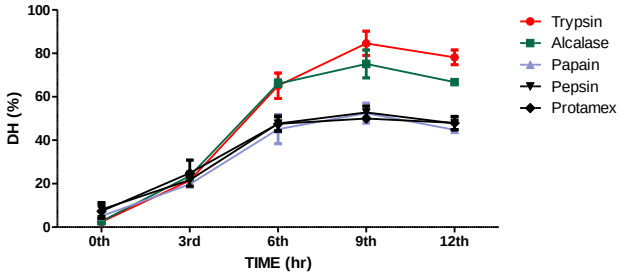

Figure 1. It shows the percentage of degree of hydrolysis of Oliva oliva visceral mass with five digestive enzymes (mean ± SD, n=3), *P>0.05.

Anti-inflammatory and Anti-Oxidant Activity of CCM Peptide

The anti-inflammatory activity is quantified by albumin denaturation assay (AD) and HRBC membrane stabilization assay. Current results show that in AD assay the alcalase 6th, 9th and 9th hr hydrolysate has higher inhibitory activity of 67.20 ± 0.88, 76.55 ± 0.37 and 69.84 ± 1.14 percentages respectively. When compared with other hydrolysate inhibitions the 12th hr alcalase shows higher activity **(Figure 2a).** The diclofenac potassium is used as standard drug (95.97 ± 0.27 %). The inflammation may also leads to the lysosomal membrane rupturing hence HRBC membrane is performed because the RBC membrane are similar to lysosomal membrane (Babu, Pandikumar and Ignacimuthu 2011). Results shows that the 9th hr alcalase hydrolysate of CCM shows a higher HRBC membrane stabilization assay about 69.03 ± 1.04 % **(Figure 2b).** The diclofenac potassium is used as standard drug (93.77 ± 0.99 %).

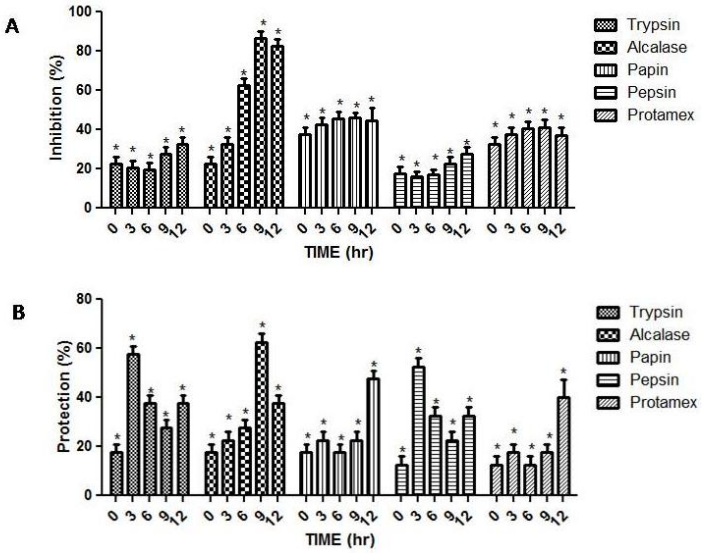

Figure 2. The AD and HRBC assay of different time variables during enzyme hydrolysis are shown in A and B respectively (mean ± SD, n=3), *P>0.005.

The DPPH is performed since the DPPH free electron is paired off (Taheri et al. 2014). The anti-oxidant capacity of CCM peptide shows that the 9th hr hrydrolysate has more activity when compared with

other hydrolysate **(Figure 3a).** This can be due to the presence of alanine and glycine amnioacid (Hajfathalian et al. 2018; Taheri et al. 2014). Hydroxyl radicals are the major reactive oxygen species (Pavithra and Vadivukkarasi 2015). The current results show the similar activity with higher anti-oxidant activity at 9th hr hydrolysate **(Figure 3b).** Some aminoacids detected also have the ability to chelate pro-oxidative transition metals, thus favoring the reduction and deactivation of OH˙ free radicals (Hajfathalian et al. 2018). All these facts could explain the dependency between OH˙ scavenging activity and concentration.

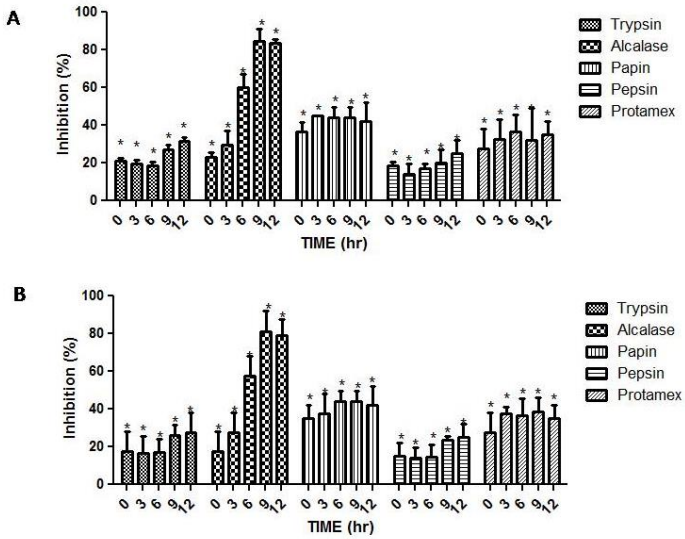

Figure 3. The DPPH and hydroxyl assay of different time variables during enzyme hydrolysis are shown in A and B respectively (mean ± SD, n=3), *P>0.005.

Purification and Sequencing of Active Fraction

The active hr sample was purified using the gel filtration chromatography **(Figure 4)** and the fraction was subjected to amino acid sequencing and molecular weight analysis using LC-MS/MS. The sequence was obtained as HPAEDR (723.76 Da). Further, for *in vitro* and *in vivo* studies the peptide derived from CCM was synthetically designed with 98% purity (PhtdPeptides Co., Ltd. Zhemgzhou, China) **(Figure 5).**

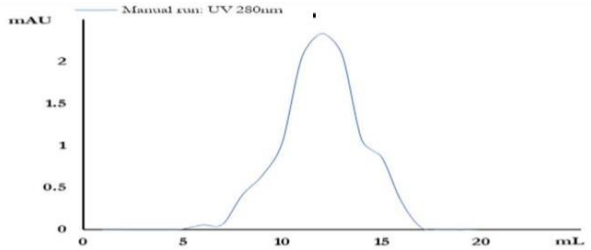

Figure 4. The gel filtration chromatogram of the 9th hr alcalase hydrolysate.

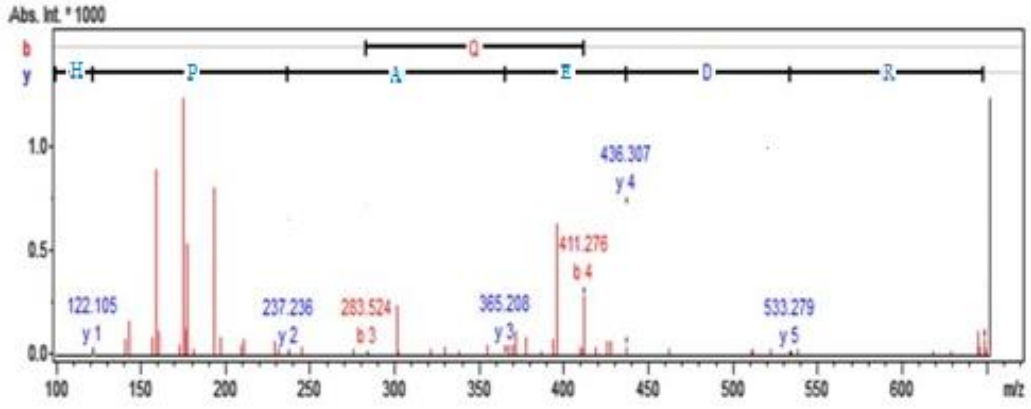

Figure 5. The amino acid sequencing of purified fractions and its molecular weight.

Physiochemical Properties

The functional property relies on the structure and amino acid sequences of the protein. The results obtained show that, an increase in solubility was observed between pH (4-10) up to 84.37 ± 1.03% **(Figure 6).** The observed increase in solubility is due to

amino acid serine which is reported earlier as an efficient amino acid regarding to solubility of protein (Trevinoet al., 2007)**.** Similarly, the emulsifying ability also relies on the structure and amino acid sequences of the protein. Another study shows that the foaming property are also responsible for the pH change and said that the pH (6-10) is steady in foaming property (Majidiyan et al. 2022). Additionally, the emulsification activity are active at pH 5 and its stability varies based on the pH (Ma et al. 2018).

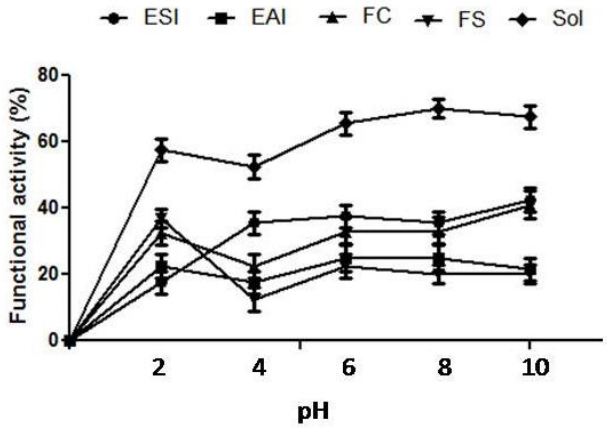

Figure 6. The functional properties of Oliva oliva veseral mass derived peptide (mean ± SD, n=3), *P<0.05

Cytotoxisity and Pro-Inflammatory Cytokines Expression

The cell viability of the drug treated cells are generally evaluated using MTT assay whereas, the current results on MTT assay shows that the cell viability was more than 80% **(Figure 7)** and hence the purified peptides were not toxic to the macrophage RAW264.7 cells. Similarly, on another study conducted using *sargassum polycystum* shows higher cell viability**.** Additionally, a anti-inflammatory peptide derived from *arca subcrenata* shows lower cell toxicity on macrophage RAW264.7 cells (Chen et al. 2017; Li et al. 2014).

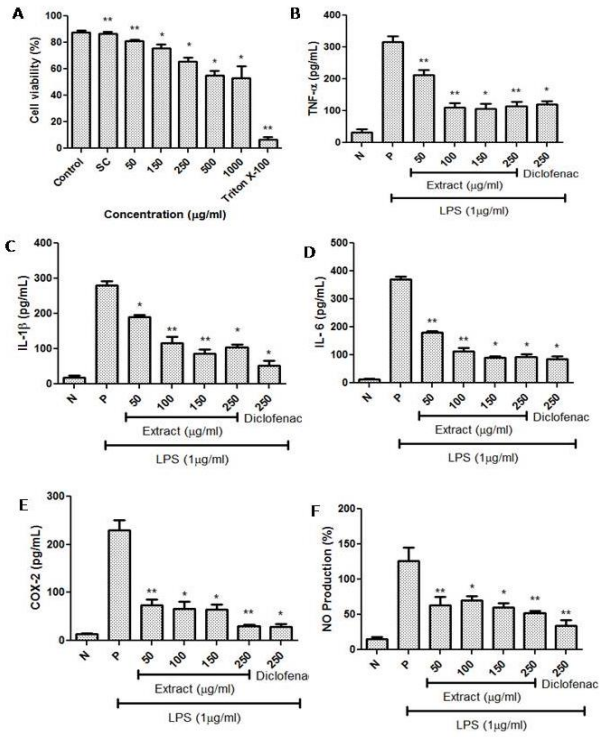

Figure 7. Cell viability (A) and anti-inflammatory activity of CCM peptide over (B) TNF-α, (C) IL-1 β, (D) IL-6, (E) COX2 and (F) NO production inLPS-induced RAW264.7cells, where N and Prepresent negative and positive control (mean ± SD) and there is significant difference, P< 0.05

The pro-inflammatory cytokines expression analyzed at a dosage of 50,100 and 200 µg/ml respectively. The results show that increase in the concentration of peptides increases the COX-2 inhibition **(Figure 7).** It is found that comparatively, the 200µg/ml and higher concentration are effective in inhibition than the lower concentration. Further, the studies carried out on oyster and Yan-Hou-Qing shows the similar activity on COX-2 inhibition (Hwang et al. 2012; Ray et al. 2019). Additionally, the expression of TNF-α and NF-KB of peptide treated Zebrafish larvae is studied **(Figure 8).**

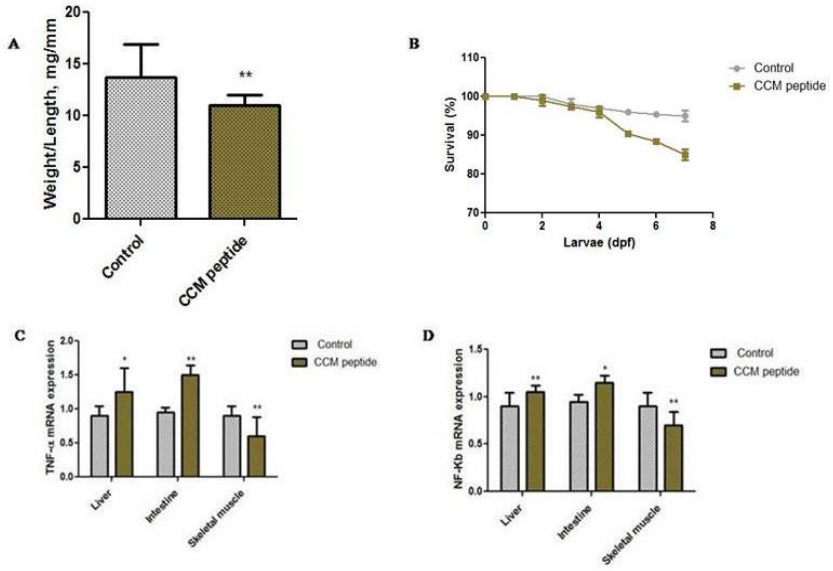

Figure 8. The peptide reduced the weight (A) of Zebrafish. It has a considerable amount of mortality (B) throughout the experiment. The C and D shows the gene expression (hadhaa and hadhb) in the Zebrafish liver, intestine and muscle of the atherosclerotic Zebrafish against fish oil Zebrafish group.

CCM Peptide Downregulated the Emergence of Plaques in Atherosclerotic Zebrafish

Figure 9 shows that zebrafish of the AC group showed a huge amount of deposition of lipids near the vascular area, but there was no change in the control group. Interestingly, the addition of 1 mg/l and 10 mg/l CCM peptide to the bath significantly reduced lipid deposition in Zebrafish larvae compared to the AC group. There were no significant differences between the AS (atherosclerotic) group and the 0.1 mg/L peptide group.

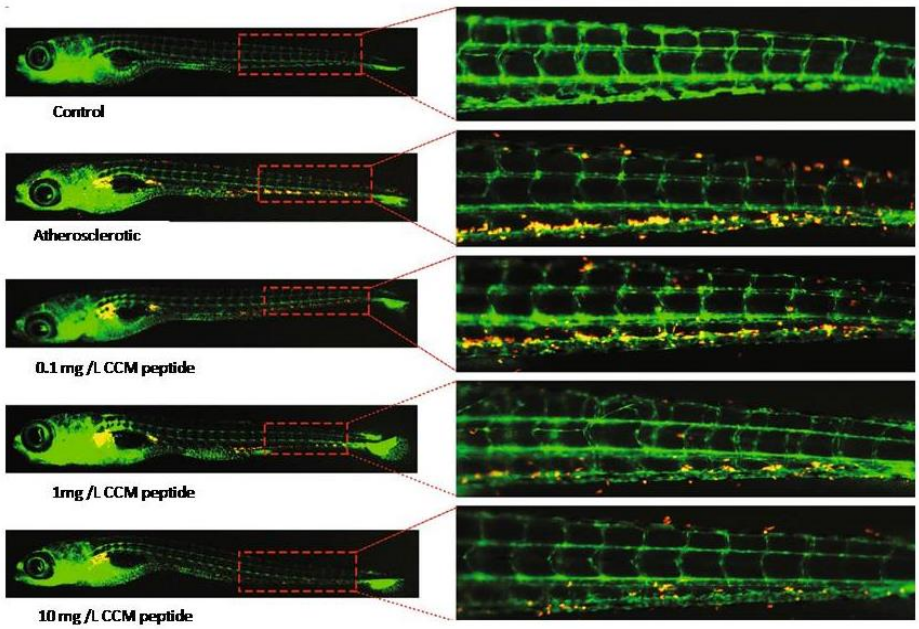

Figure 9. Larvae induced with 4% red florescence attached high cholesterol food (HCF) for 10 days where, the dotted box represents the crucial area of accumulated lipids.

CCM Peptide Improved Liver Metabolism and Oxidative Stress in Atherosclerotic Zebrafish

Compared to the manage organization, the AS organization exhibited a enormous growth in lipid degrees, which have been considerably decreased with the aid of using 1mg/L and 10mg/L CCM peptide **(Figure 10(a)). Figures 10(b) and 10(c)** exhibit that TC and TG degrees have been additionally considerably reduced in AS zebrafish dealt with with 1mg/L and 10mg/L peptide, however now no longer with 0.1mg/L peptide. In conclusion, peptide should successfully lessen lipid degrees in AS zebrafish.

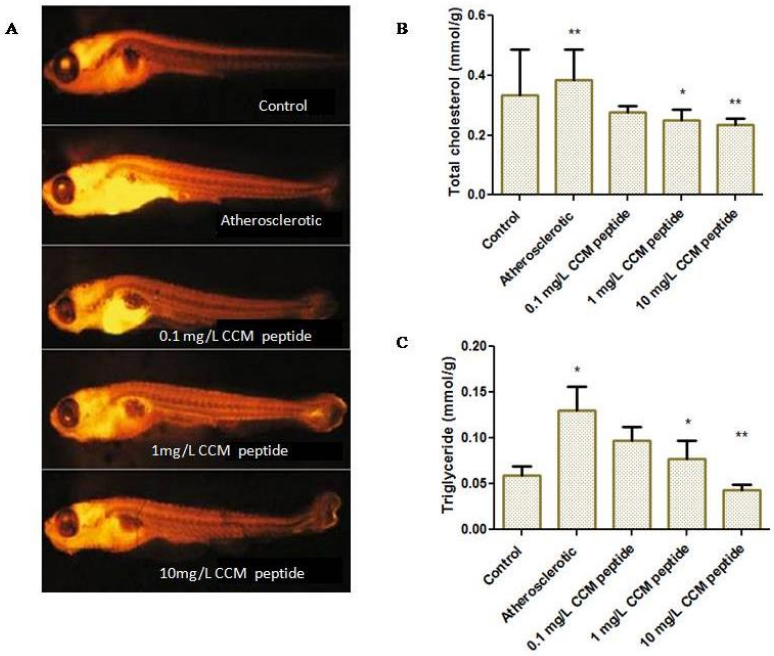

Figure 10. Nail red staining images of control anpeptide treated (A) at various concentration (exposed for a period of 10 days). Total cholesterol (B) and Triglycerides (C) of Zebrafish. Significance was calculated by one-way ANOVA and unpaired t-test using Graphpad prizom.

CCM Peptide Protected Against Lesion in Atherosclerotic Zebrafish

Neutrophil is widely regarded to be the principal cell type responsible for tissue damage and acute lesion. To determine the characteristics of the lesion response in the early stages of atherosclerosis, we first created a genetically modified Zebrafish line that upregulated the GFP of neutrophils in transgenic mpx: "EGFP" zebrafish. We discovered a significant Upregulation of green fluorescentlylabeled neutrophils in vascular locations in atherosclerotic Zebrafish larvae group **(Figure 11)**. In contemplation to determine the benefit of fish oil as a therapeutic medication that inhibits GFP-neutrophils transhumance and lesion response, we administered varying dosages of CCM peptide to Zebrafish larvae fed an HCD diet. Both the 1mg/L and 10mg/L concentrations of CCM peptide resulted in a reduction of neutrophils in the tails of zebrafish larvae, however the 0.1mg/L concentration was unaffected. In conclusion, CCM peptide protected zebrafish from oxidative damage and inflammation.

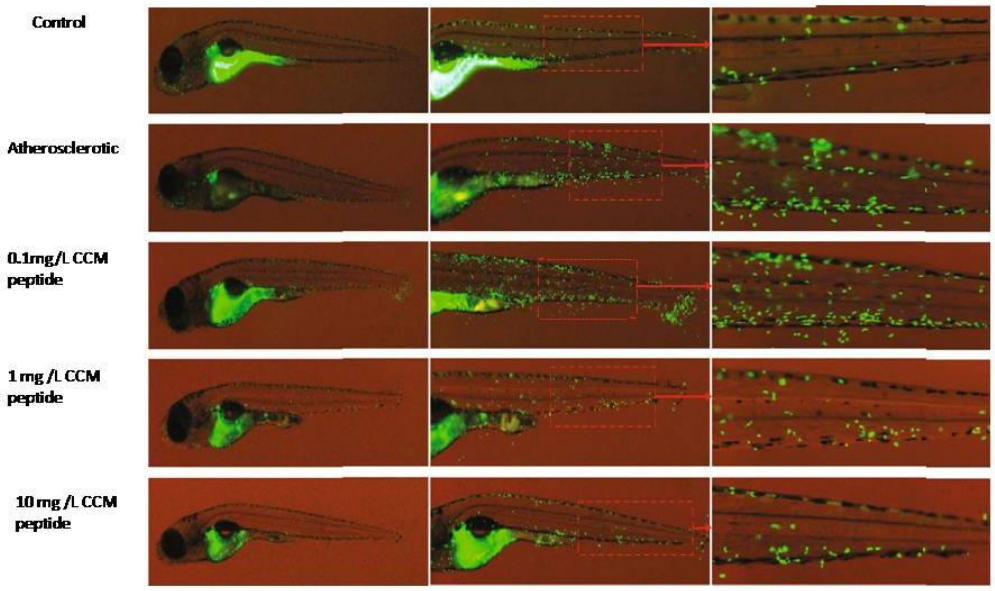

Figure 11. Anti-inflammatory effect of peptidein HCF induced atherosclerosis on Zebrafish (EGFP) larvae. The green fluoresce represents the inflammation level of neutrophils.

CONCLUSIONS

The *Catla catla* muscle(CCM) derived peptide is isolated and purified using Ultrafiltration and Gel filtration chromatography. By the anti-inflammatory assays (AD and HRBC) the active hour is identified as 9th hr alcalase hydrolysate. Additionally, the fractions are purified and the sequence is found using LC/MS-MS as HPAEDR with a molecular weight of 723.76 Da. Further, in vitro studies were carried out using LPS induced inflammation on RAW264.7 macrophage cells against synthetically designed peptide and found that the peptide is non-toxic and posses ability to suppress a pro-inflammatory cytokine in increased quantity. Additionally, it exhibits reflex effects in HCD-induced hyper-lipidemia, lesion and oxidative stress and inhibits formation of atherosclerotic plaque. Hence this study suggests that the CCM derived peptide may be work as a supplementary medicine to treat chronic inflammatory diseases and may posses cholesterol reducing effect.

Code Availability: No code availability

Data Availability: Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Authors' Contributions: Sabarinathan S and Revathy LR have contributed in all aspects of research and manuscript preparation under the guidance of Janet Rani R.

Competing Interests: The authors declare that there are no competing interests.

Funding: The authors declare that nofunding was received to assist this research

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