

Original Research Article

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Isolation and Purification of Protein from Skin and Muscles Homogenate of *Labeo rohita* (Rohu)

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ABSTRACT

India is endowed with an extensive network of fisheries resources; a long coast line of 1100 km along the Arabian Sea, supports a wide range of fish species with economic value and nutritional importance. Approximately 180 species of fish are found in fresh waters of India, majority of which are edible Antimicrobial proteins and peptides had been found from a wide variety of organisms in the last few years. These molecules have attracted much research interest because of their biochemical diversity, broad specificity on anti-viral, anti-bacterial, anti-fungi, anti-protozoan parasites, anti-tumoural, and wound-healing effects. Antimicrobial proteins and peptides play key roles in innate immunity. They interact directly with bacteria and kill them. The present study was performed in order to isolate and purify the protein from skin and muscles homogenate of *Labeo rohita* (Rohu). Different studies are already performed which showed that bacteria and virus have caused high mortality in *Labeo rohita* cultures, but the endogenous antimicrobial peptides and proteins have not been explored till yet or less evidences are reported. As per the results observed, an antimicrobial component was obtained from the skin and muscles homogenate of *L. rohita*. It was observed that when the skin and muscles homogenate was digested with trypsin (proteolytic enzyme), its antimicrobial activity was lost, which confirmed that the antimicrobial component is a protein. The antimicrobial protein was further purified from the skin and muscles homogenate of *Labeo rohita* by successive ion-exchange and gel filtration chromatography. The protein isolated was demonstrated to be single protein band by SDS-PAGE, with the apparent molecular weight of 46 KDa. The crude protein content in the mixed homogenate of the fish was found to be 20.5%. Although different feed content may also vary the nutritional significance of different biomolecules in the fish which is also another part of study. The protein fraction isolated was evaluated for antimicrobial activity. The protein fraction exhibited antimicrobial activity both for the Gram-positive bacteria, *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis*, and for the Gram-negative bacteria, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Pasteurella multocida*, *Aeromonas hydrophila*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Except *A. hydrophila*, *P. aeruginosa*, and *E. coli* (MIC>19 mol/L), most of the tested Gram-negative bacteria were sensitive to protein fraction (MIC<18 mol/L). Interestingly, the protein fraction showed potent antimicrobial activity against Gram-positive bacteria, *S. aureus* (MIC 5-10 mol/L) but comparatively weak antimicrobial activity against *M. luteus* and *B. subtilis*. The study thus suggests the presence of antimicrobial amino acids in the protein fraction which are needed to be isolated and characterization for further studies.

Keywords

Antimicrobial activity, Fresh water, *Labeo rohita*, Protein fraction.

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Introduction

Fish meat possesses high nutritional quality and is therefore a particularly recommended human dietary component. Information concerning the chemical and fatty acid composition of freshwater fishes is valuable to nutritionists who are interested in finding sources of low-fat, high protein foods, with desirable fatty acid compositions and acceptable amount of total cholesterol. Fish meat contains biologically active protein which is characterized by a very favorable composition of amino acids, a high omega-3 polyunsaturated fatty acid content such as eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic (22:6 n-3, DHA), and fat-soluble vitamins as well as it represents a good source of micro- and macro-elements. Antimicrobial proteins and peptides were obtained from a wide variety of organisms. These molecules have attracted much research interest because of their biochemical diversity, broad specificity on anti-viral, anti-bacterial, anti-fungi, anti-protozoan parasites, and even anti-tumoural or wound-healing effects (Zasloff, 1987; 2002). Compounds with broad antimicrobial activities are thought to be especially important for fish, as their adaptive immune system is structurally simpler than that of mammals or amphibians, and is not fully effective in young fry or at low environmental temperature (Silphaduang and Noga, 2001). The skin epithelium and other mucosal surfaces of fish are rich in anti-microbial peptides (Bergsson *et al.*, 2005). The present study is in need to determine the important nutritional components present in fish, *Labeo rohita* (Rohu) as most of the population of India residing near the banks of the rivers and specific communities utilizes fish as their regular diet. The study was performed to focus on the nutritional and pharmacological components of fish which makes the fish as an important nutritional and dietary supplement. The study was performed

to isolate and determine the antimicrobial nature of the protein in skin and muscles homogenate of *Labeo rohita*.

Materials and Methods

Collection of samples

Sampling of farm cultivated and river water, *Labeo rohita* (Rohu) fish species was performed for the comparison of fatty acid composition from Doiwala region of Dehradun (U.K).

Preparation of grouper skin homogenate

Healthy individuals of *Labeo rohita* (weight range 500–600 g) were procured. The individuals were killed by stabbing the brain with a sterilized knife, after 12 h. Their scales were scraped gently and skins were peeled and washed in 50 mMol/L Tris-HCl buffer (pH 7.8, containing 5 mMol/L EDTA and 0.1 mol/L NaCl). The skins and muscles were immersed immediately in liquid nitrogen. Then, the frozen skins and muscles were ground into powder with a mortar and pestle under liquid nitrogen, and homogenized in the same buffer. The homogenate was centrifuged at 15,000 g for 30 min. The supernatant was collected, lyophilized and stored in -80°C until use.

Purification of antimicrobial protein from skin and muscles homogenate of *Labeo rohita*

The skin and muscles homogenate was purified by ion exchange chromatogram and gel filtration chromatogram. Briefly, the lyophilized skin homogenate was dissolved in 20 mL 50 mMol/L Tris-HCl (pH 7.8, containing 5 mMol/L EDTA) and dialyzed against the same buffer for 24 h at 4°C (The molecular weight cut-off of the dialysis tubing was 3,500 D). Then, the sample was

loaded in DEAE-Sephadex A-50 ion exchange column (2.6 cm×30 cm). The elution was performed at a flow rate of 30 mL/h with a linear NaCl gradient. The fractions were lyophilized and applied to a Sephadex G-75 (superfine) column (2.6 cm ×100 cm) equilibrated with 50 mMol/L Tris-HCl (pH 7.5, containing 5 mMol/L EDTA and 0.1 mol/L NaCl). Elution was achieved with the same buffer at a flow rate of 9 mL/h, collecting fractions of 3 mL per tube. The protein fractions was collected, dialyzed against 50 mMol/L Tris-HCl (pH 8.8, containing 5 mMol/L EDTA) for 24 h at 4°C, and loaded on a Q-Sepharose (high performance) column (2.6 cm ×30 cm) pre-equilibrated with the same buffer. A linear NaCl gradient of 0–0.5 mol/L was employed to elute the proteins. The elution was performed at a flow rate of 30 mL/h with a linear NaCl gradient, collecting fractions with antimicrobial activity of 5 mL per tube.

Protein concentration

The protein concentration was determined by the Lowry method according to the procedure described (Gerhardt *et al.*, 1994). In this procedure, the proteins were first pretreated with copper ions in an alkali solution. The aromatic amino acids in the treated sample reduced the phosphomolybdic-phosphotungstic acid present in the Folin's reagent. Since the endpoint of the reaction has a blue color, the amount of protein in the sample could be estimated by reading the absorbance using a spectrophotometer (Systronics- UV-VIS spectrophotometer) at 750 nm.

Electrophoretic studies

SDS-PAGE was performed as reported by Laemmli (1970). For SDS-PAGE, samples were pretreated in 2.5% SDS alone (nonreducing conditions) or in 2.5% SDS and

5% mercapto ethanol (reducing conditions) for 5 minutes at 100°C. Gels were stained with 0.1% Coomassie brilliant blue R-250.

Antimicrobial assays

Different bacterial strains were selected for this study, Gram-negative bacteria including *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Pasteurella multocida*, *Vibrio fluvialis*, *Aeromonas hydrophila*, *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive bacteria including *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis*. Strains were grown on broth nutrient medium. Antimicrobial activity was monitored by a liquid growth inhibition assay. Briefly, logarithmic phase bacterial cultures were diluted in the broth [1% (w/v) bacterio-tryptone, 0.9% (w/v) NaCl] to an A_{600} of 0.001, which is approximately equivalent to 10^5 CFU/ml. Diluted bacteria (90 μ L) were mixed with 10 μ L of either water (control) or purified protein in wells of a micro-titration plate. After overnight incubation at 25°C, the bacterial growth was monitored by measuring the change in the absorbance of the culture with a microplate reader at 600 nm (Casteels *et al.*, 1993).

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the antimicrobial protein was determined as follows. Briefly, bacteria were incubated in Todd Hewitt broth [50% (w/v) beef heart infusion; 2% (w/v) peptic digest of animal tissue; 0.2% (w/v) dextrose; 0.2% (w/v) NaCl; 0.04% (w/v) Na_2HPO_4 ; 0.25 (w/v) Na_2CO_3] in the presence of 2-fold serial dilutions of sample (final concentration 1.25–80 μ Mol/L). Bacterial growth was monitored by a liquid growth inhibition assay. MIC was expressed as a range of the highest

concentration of purified protein at which bacteria were able to grow and the lowest concentration that the bacterial growth was completely inhibited. All assays were performed in duplicate (Lauth *et al.*, 2002).

Proteolytic digestion of homogenate

Susceptibility of the antimicrobial activity of crude skin and muscles homogenate to proteolytic digestion was determined by incubation of 4.5 mg skin homogenate (containing 1 mg proteins) with 250 µg/L crystalline trypsin for 60 minutes at 37°C. Antimicrobial activities of the skin and muscles homogenate before and after protease (trypsin) treatment against the similar bacterial cultures were determined.

Results and Discussion

The present study suggested the isolation and separation of protein from skin and muscles homogenate by centrifugation (Figure 1). Further the proteins were purified via ion-exchange and gel chromatography (Figure 2). The molecular weight of the protein fraction isolated and purified was determined. The concentration of the purified protein was

determined as 20.5 % of the total skin and muscles homogenate prepared as determined by quantitative method. The results of the electrophoretogram showed the molecular weight of the protein as 46 KDa (Figure 3).

The antimicrobial activity of the protein was determined against *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Pasteurella multocida*, *Vibrio fluvialis*, *Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis* at 500 mg/ml. The results were found to be very surprising as the protein fraction was found to have antibacterial activity against most of the pathogens studied.

The results follows the order viz. *V. parahaemolyticus* (56.0 mm) > *P. multocida* (48.0 mm) > *V. alginolyticus* (45.0 mm) > *S. aureus* (42.0 mm) > *V. fluvialis* (38.7 mm) > *M. luteus* (21.0 mm) > *B. subtilis* (18.5 mm). No antibacterial activity was found against *A. hydrophila*, *E. coli* and *P. aeruginosa*. It was also observed that, when treated the results are shown in table 1 and figure 4 (a); (b). The results of MIC are shown in table 2 and figure 5.

Table.1 Antimicrobial activity of protein fraction purified as such and Protein fraction treated with trypsin

Protein fraction purified	Diameter of zone of inhibition (mm) against pathogens									
	VA	VPH	PM	VF	AH	ECO	PA	SA	ML	BS
Protein fraction purified (500 mg/ml)	45.0	56.0	48.0	38.7	NA	NA	NA	42.0	21.0	18.5
Protein fraction purified after treatment with trypsin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

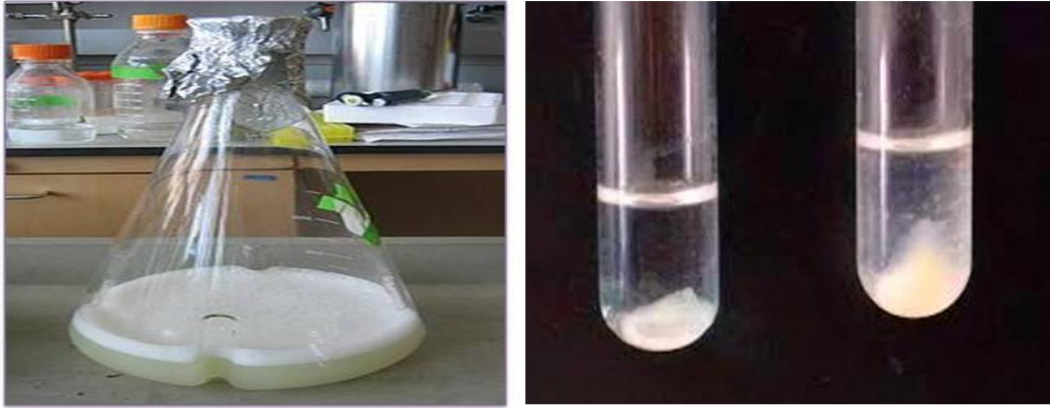
**V. alginolyticus* (VA), *V. parahaemolyticus*, (VPH) *P. multocida* (PM), *V. fluvialis* (VF), *A. hydrophila* (AH), *E. coli* (ECO) and *P. aeruginosa* (PA), *S. aureus* (SA), *M. luteus* (ML) and *B. subtilis* (BS); NA, No activity

Table.2 MIC of protein fraction purified as such and protein fraction treated with trypsin

Protein fraction purified	MIC (mol/liter)									
	VA	VPH	PM	VF	AH	ECO	PA	SA	ML	BS
Protein fraction purified	12	10	13	15	19	25	27	11	29	31
Protein fraction purified after treatment with trypsin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

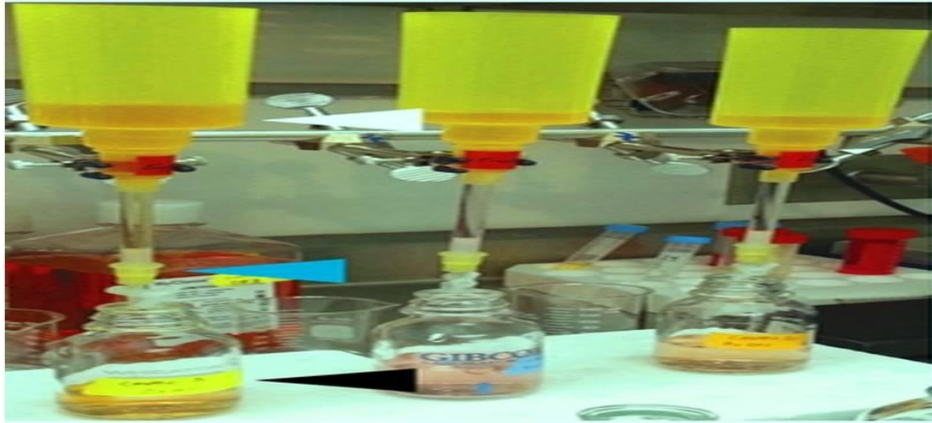
**V. alginolyticus* (VA), *V. parahaemolyticus*, (VPH) *P. multocida* (PM), *V. fluvialis* (VF), *A. hydrophila* (AH), *E. coli* (ECO) and *P. aeruginosa* (PA), *S. aureus* (SA), *M. luteus* (ML) and *B. subtilis* (BS)

Fig.1 Separation of proteins from skin and muscles homogenate of *Labeo rohita*



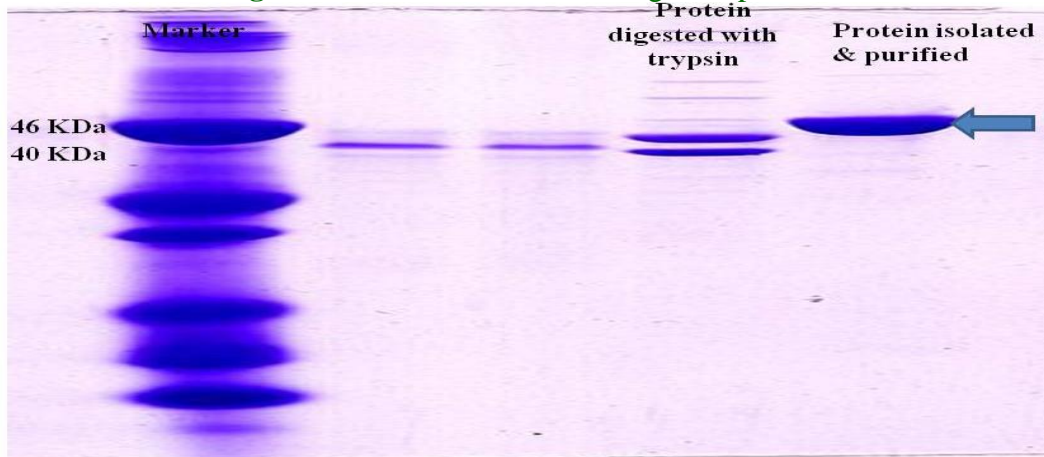
Homogenization and centrifugation of skin and muscles of *Labeo rohita*

Fig.2 Purification of protein isolated via Ion exchange and Gel chromatography



Purification of protein by Ion-exchange and gel chromatography of protein fraction from skin and muscles homogenate of *Labeo rohita*

Fig.3 SDS PAGE of intact and digested protein



SDS PAGE electrophoretogram of protein purified and protein digested with trypsin

Fig.4 (a) Graphical representation of antimicrobial activity of protein fraction as such and Protein fraction treated with trypsin

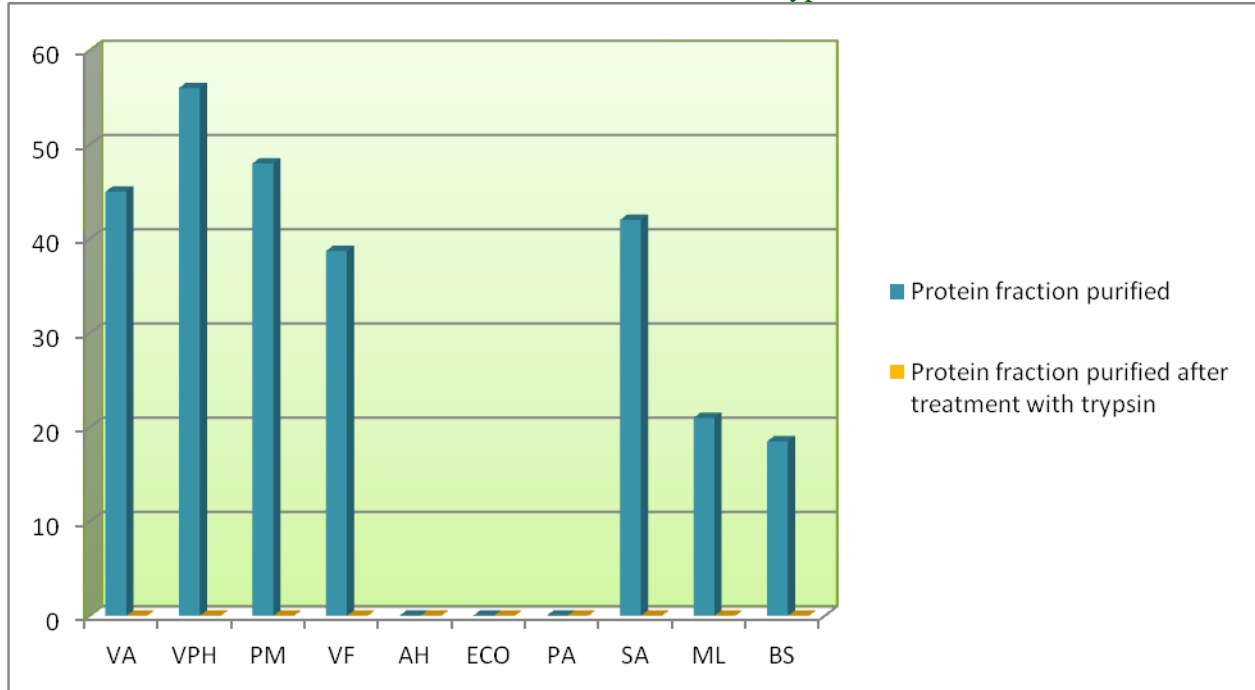
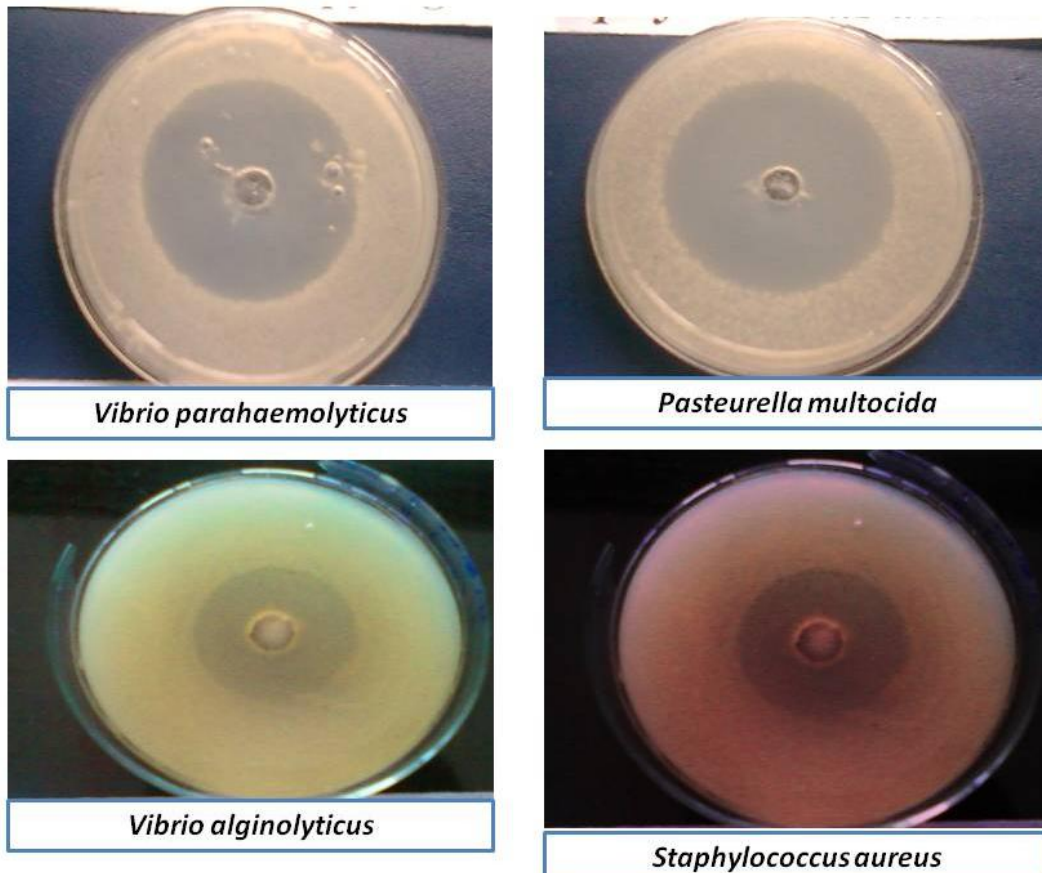


Fig.4 (b) Antimicrobial activity of protein fraction against pathogenic microbes



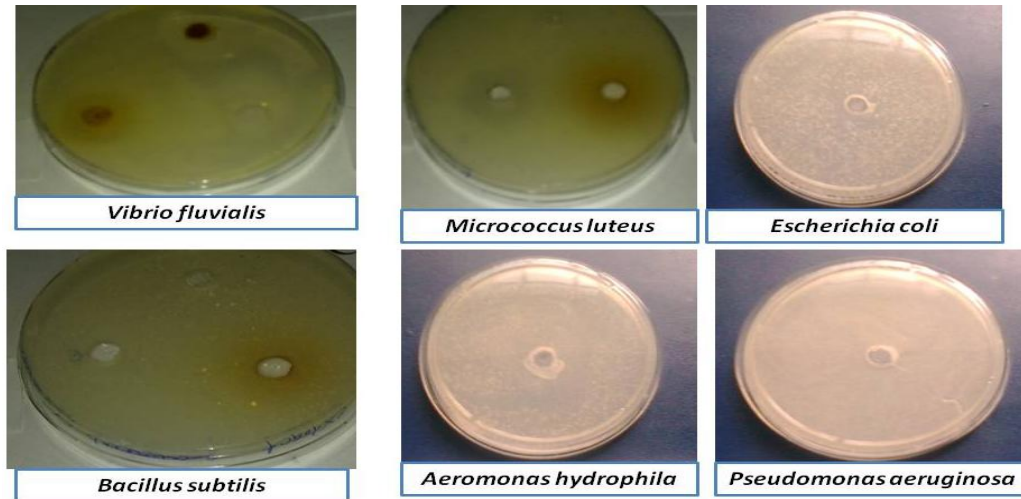
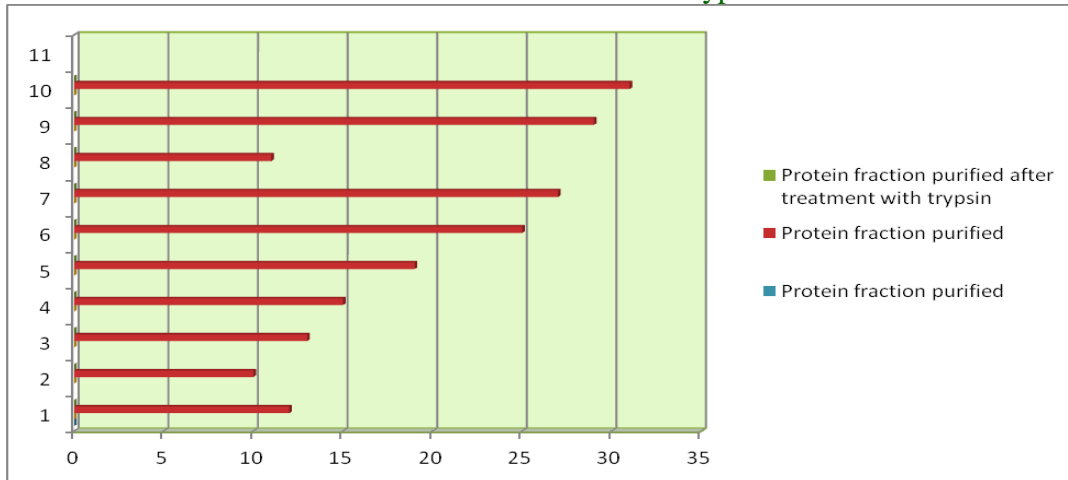


Fig.5 Graphical representation of MIC values of protein fraction as such and Protein fraction treated with trypsin



The previous studies on skin mucus describing antimicrobial nature was illustrated (Khondoker *et al.*, 2014). The bactericidal proteins from skin mucus and skin extracts from fresh water fish's viz. *Clarias batrachus* and *Tillapia mossambicus* were determined (Ponmanickam *et al.*, 2013). The proteins in *Piaractus mesopotamicus* (Pacu) were determined as an indicator of innate immunity (Biller-Taka-hashi *et al.*, 2013). Recent studies were performed on *Labeo rohita* (Rohu) after exposure to phenolic compounds (Gaur *et al.*, 2016a; 2017b).

The study thus suggests the presence of antimicrobial amino acids in the protein

fraction of *Labeo rohita* which are needed to be isolated and characterized for further studies. The present study suggests that, the protein is most efficient antimicrobial agent against dreadful pathogens. The study provides a path for exploration of such antimicrobial agents from such fresh water fishes and other aquatic fauna.

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