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ANTIBACTERIAL AND HAEMOLYTIC ACTIVITIES OF TISSUE PROTEINS OF THE MAJOR CARP, *LABEO ROHITA* (HAMILTON)

[1]Sundaramoorthy, M., [1]Gomathi, S.,[1]Kannaki, S.& [2]Saravanan,T.S.
[1]PG & Research Department of Zoology, AVC College (Autonomous), Mannampandal, TN, India
[2]PG & Research Department of Zoology, Jamal Mohamed College, Tiruchirappalli, TN, India

ABSTRACT
The Antimicrobial proteins(AMPs) of kidney, liver, heart and gills of *Labeo rohita* were partially purified by acid extraction and ammonium sulphate precipitation. The proteins extracted from the tissues were estimated and found to be 106.5 mg, 64.0 mg, 160.0 mg and 48.2 mg/g.wet.wt respectively. Out of the four organs, the heart tissue has yielded the highest amount of protein i,e 160 mg /g wet .wt. All the four proteins exhibited bactericidal effects for most of the test organisms. The maximum diameter of inhibitory zone (11 mm) was observed for the kidney protein against *E. coli* and *P. aeruginosa.* For many bacterial strains, the MICs of all the proteins were found to be at the lowest concentration of 16 µg. Further none of the protein purified showed haemolytic effects on ABO hRBCs.

KEYWORDS: kidney, liver, heart, gills, bactericidal effects, *E. coli, P. aeruginosa*

INTRODUCTION

The serious problem associated with the use of antibiotics in treating infectious diseases is the resistance of pathogens. It is a worldwide problem in human and veterinary medicines. To date tuberculosis and pneumonia are prominent examples of once easily treated infections where drug-resistance has become a major threat now. Similarly the infection by *Staphylococcus aureus* was treated successfully with penicillin in the 1940s and 1950s. But now nearly all strains are resistant to penicillin. It is generally accepted that the main risk factor for the increase in antibiotic resistance is an extensive use of antibiotics. The antibiotic resistant genes are also distributed from one to another by natural transformation. Hence, there is an urgent need to search for new alternatives to the synthetic antibiotics. The antibacterial drugs from natural sources may be suitable to solve this problem and hence a vast study on several medicinal plants is being carried out since long ago. The antimicrobial properties of different solvent extracts of various plants have been most intensively studied (Nascimento, 2000; Lauk *et al*., 2003; Mahesh and Satish, 2008; Audipudi *et al*., 2010). However, the systemic treatment by herbal drugs has not yet developed well due to certain unknown problems found in their preparation.

The innate immune system of animals provide rapid and effective host defense against microbial invasion in a manner that is independent of prior exposure to a given pathogen (Levy, 2000). It is well known that a part of innate immunity includes some sort of proteins which take part in host defense. There is a lot of evidences for that the proteins called as Antimicrobial proteins (AMPs) serve as host defense substances by direct antimicrobial activity (Boman, 1995; Goldman *et al*., 1997; Bals *et al*., 1999; Wilson *et al*., 1999; Bals, 2000). They are distributed in all species of life, ranging from plants and insects to animals including mollusks, crustaceans, amphibians, birds, fish, mammals and humans. (Hancock and Lehrer, 1998). Many of these proteins have a broad spectrum of activity against bacteria, fungi, parasites and viruses. Host defense peptides (HDPs) produced by the epithelial cells and phagocytic cells, are an important component of innate immune defense at mucosal surfaces of mammalian and non.mammalian species (Koczulla and Bals, 2003).

Antimicrobial peptides show promise for becoming the next major group of antibiotics (Fleury *et al*., 1996). These peptides may prove more difficult for microorganisms to develop resistance based on their mode of action. Instead of interacting with receptors or enzymes, which are more easily changed by mutations, these peptides interact with negatively charged phospholipids and lipopolysaccharides (LPS) of the membrane (Fredericks and Dankert, 2000). In recent years, the use of such host defense proteins to treat the infectious diseases has attracted the researchers very much.

As most of these peptides have been identified only from the blood cells, epithelial cells of reproductive and respiratory tracts and skin cells (Diamond *et al*.,1991; Gazzano *et al*., 1992; Bals, 2000; Avellar, 2004), their industrial preparation is found to be somewhat tedious. Hence, it is inevitable to screen the freely available organs of animals for the presence of AMPs at high concentration. Recently for the first time, Sundaramoorthy and Saravanan (2010) have investigated the antibacterial properties of goat and chicken heart tissues. A 13 KDa non-hemolytic antibacterial peptide against *pseudomonas aeruginosa* was also purified from chicken heart tissues (Sundaramoorthy and Saravanan, 2011).

The presents study aims to prepare crude antibacterial proteins by chemical extraction from the tissues of liver, heart, kidney and gills of *Labeo rohita* and to check their antibacterial activities against certain selective human pathogenic bacteria like *Escherichia coli*, *Staphylococcus*

aureus, klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi'A' and *Salmonella typhimurium* species isolated from clinical samples in the view of generating natural antimicrobial proteins/peptides at considerable quantity directly from the organs of fishes as they are easily available for human consumption.

MATERIALS AND METHODS

Animal collection

The fish, *Labeo rohita* were collected from a fish pond located at Kuthalam, Mayiladuthurai, Tamilnadu, India and transported to the laboratory in a plastic bag with sufficient amount of fresh water.

Tissue preparation

The animals were sacrificed and the organ (kidney, liver, heart and gills) were collected and washed well with water and normal saline .Then they were minced into small pieces and the blood clots were removed by repeated washing with tap water and normal saline.

Acid extraction

100 mg of tissue of each organ was homogenized in 5 ml of 10% acetic acid and centrifuged at 5000 rpm. The filtrate was collected and boiled at 100°C for 10 minutes. Again centrifuged at 6000 rpm for 10 minutes and the filtrate containing the acid extracted proteins was collected in separate tubes. The tubes were stored at -20°C until use (Matutte *et al*., 2000).

Ammonium suphate precipitation

1ml of acid extract was mixed with 1ml of 30% ammonium sulphate and allowed to stand at room temperature for 10 minutes. Then centrifuged at 6000 rpm for 10 minutes and the supernatant was discarded. The precipitate was dissolved with 4 ml of sterile water and stored at -20°C for analysis.

Protein estimation

The concentrations of the dissolved proteins were estimated by adopting the procedure of Lowry *et al*.,(1951).

Collection of bacterial isolateds

Seven bacterial isolates, *E.coli, S. aereus, K. pneumonia, P. aeruginosa, S. typhi, S. paratyphi'A'* and *S. typhimurium* were procured from Microbiology Laboratory, KAPV Medical college, Thiruchirapalli, Tamilnadu, India. The isolates were also confirmed by gram staining and standard biochemical tests and maintained in semisolid medium.

Antibacterial assay

The antibacterial activity of the 30% ammonium sulphate purified proteins was determined by standard disc diffusion method. The mid logrithmic phase of all the seven bacterial strains were suspended in 1% peptone water and the turbidity was adjusted to have 2×10^6 CFU/ml using 0.5 Mac Farland standard. Then the suspended bacterial isolates were diluted into 20 times with 1% peptone water and inoculated on to the entire surface of MHA media using sterile swabs. The surface of each medium with lawn of the testing organisms was divided into four equal quarters. Then the discs soaked with the protein solutions were gently placed at the respective areas. The plates were incubated overnight at 37°C. The diameter of zone of inhibition was measured with the help of a scale.

MIC (Minimum Inhibitory Concentration)

The minimum inhibitory concentrations of all the four protein samples were determined by Microdilution method using resazurin as an indicator of cell growth as described by Sarker *et al*, (2007). The turbidity of test organisms were adjusted to obtain 2×10^6 CFU/ml approximately using 0.5 Mac Farland standard. Then they were diluted in 200 times by 1% peptone water.

The MIC plates were prepared under aseptic conditions. A sterile 96 well plates was labeled as per the protocol designed. A volume of 200µl (1mg/ml) of protein was pipetted into the first column of the plate. To all other wells, 100µl of 1% peptone water was added. A serial dilution was performed and ensured that each well had 100µl of protein in serially descending concentration. Then 10µl of bacterial suspension was added to each well. Finally to each well, 10 µl of resazurin indicator solution (270 mg resazurin diluted in 40 ml of distilled water) was added. A positive control (Ciprofloxacin of the same concentration as that of the protein concentration) was also subjected in a separate row against an organism randomly selected for each set of organisms. The plates were covered with the lid provided and incubated for 24 hours at 37°C. The colour change was then observed visually. The colour change from purple to pink or colourless were recorded as the presence of bacterial growth where the purple means inhibition of growth. The minimum inhibitory concentration is defined as the lowest concentration of protein sample at which 100% growth inhibition occurs.

Haemolytic assay

According to Zhu *et al*, (2007), the hemolytic assay of all the four 30% ammonium sulphate purified protein samples were carried out in micro well plates. The 'A' 'B' and 'O' group blood samples were collected from student volunteers of AVC College in separate anticoagulant containers. The red blood cells were sedimented by centrifugation at 3000 rpm. The RBCs were repeatedly washed with 0.9% Nacl. The concentration of hRBCs were converted into 4% suspension with phosphate buffer solution (PBS PH.7). The wells of the first three rows were filled with 200µl (1mg/ml) of protein solution. All other wells were filled with 100µl of PBS buffer. Then the protein solution was serially diluted to ensure that each well had 100µl of protein in serially descending concentration. Then 100µl of 4% hRBCs at each group was added in each well of the respective rows. The 4% hRBCs alone and 4% hRBCs in 1% Triton 100× were used as 0% (-ve control) and 100% (+ve control) haemolysis respectively. After 2 hours incubation the button formation was recorded as absence of haemolysis. The minimum haemolytic concentration (MHC) of the protein is defined as the lowest concentration of protein at which 100% haemolysis occurs.

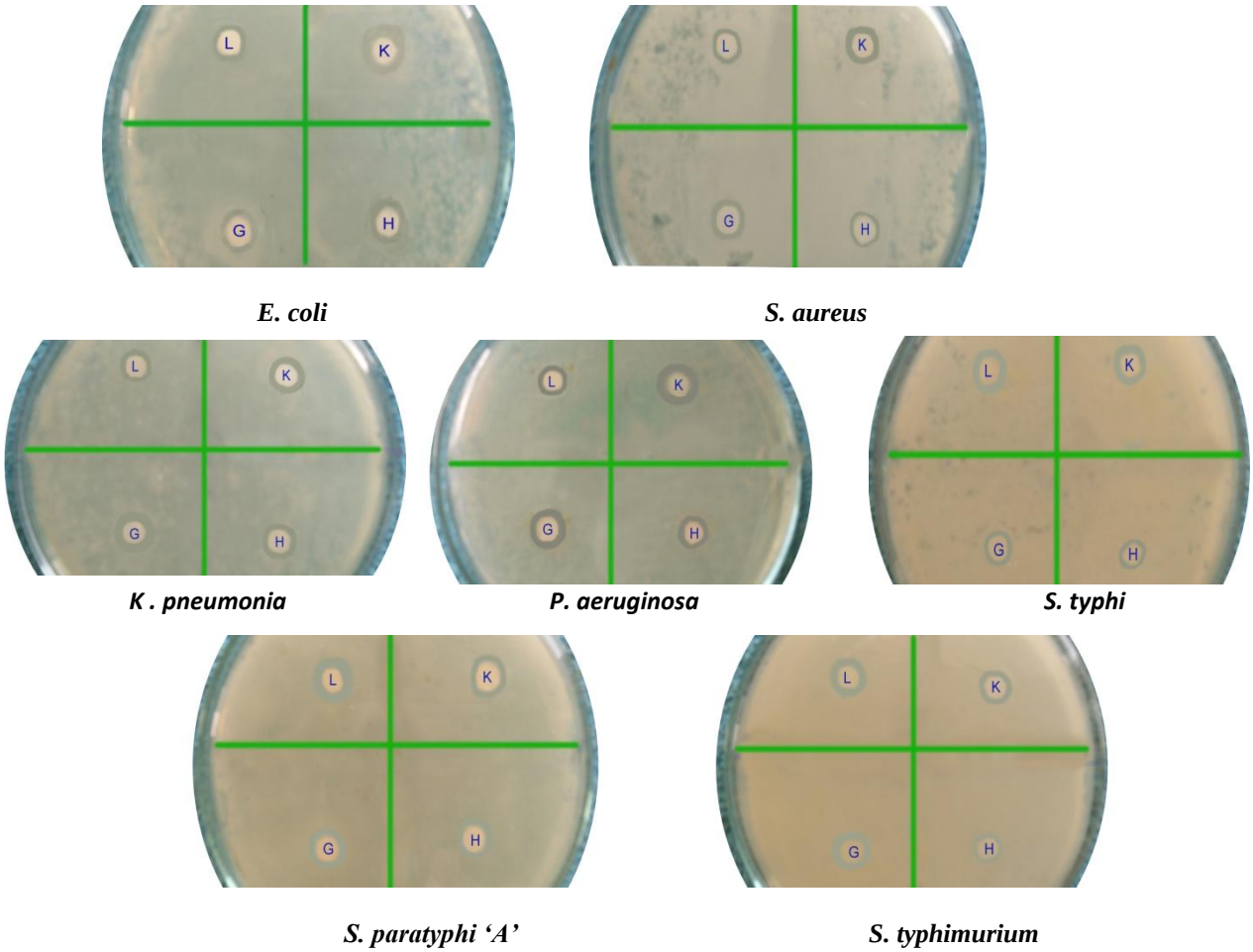
RESULTS

The expected antibacterial proteins of kidney, liver, heart and gills have been partially purified by 30% ammonium sulphate precipitation followed by acid extraction. The total amount of purified proteins were estimated and found to be 106.5 mg, 64.0 gm, 160.0 mg and 48.2 mg/ g.wet.wt. of the above mentioned organs respectively.

Antibacterial Activity

The antibacterial activity of the partially purified proteins was carried out for seven bacterial isolates. The proteins of all the four tissues showed significant bactericidal activities for most of the organisms as shown in the Fig.1. The inhibitory zones were found to be in the range of 6 - 11mm in diameter. The maximum size (11 mm) of the inhibitory zone was produced by kidney protein against two organisms, *E.coli* and *P. aeruginosa*. The general observation show that the growth of all the organisms have been inhibited by all the four tissue proteins within the above mentioned range.

FIGURE 1. Anti bacterial activities of kidney, liver, heart and gill proteins of *Labeo rohita* against different clinical isolates



Note : The alphabets K L G H indicate the discs soaked in protein solution of the respective tissues.

FIGURE 2. Minimum Inhibitory Concentrations of different tissue proteins of *Labeo rohita* against different clinical isolates

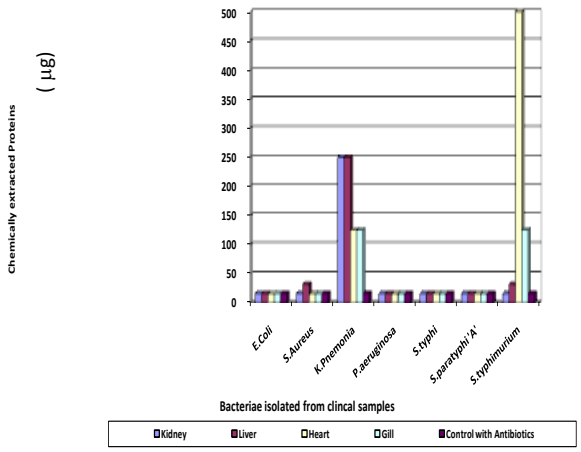
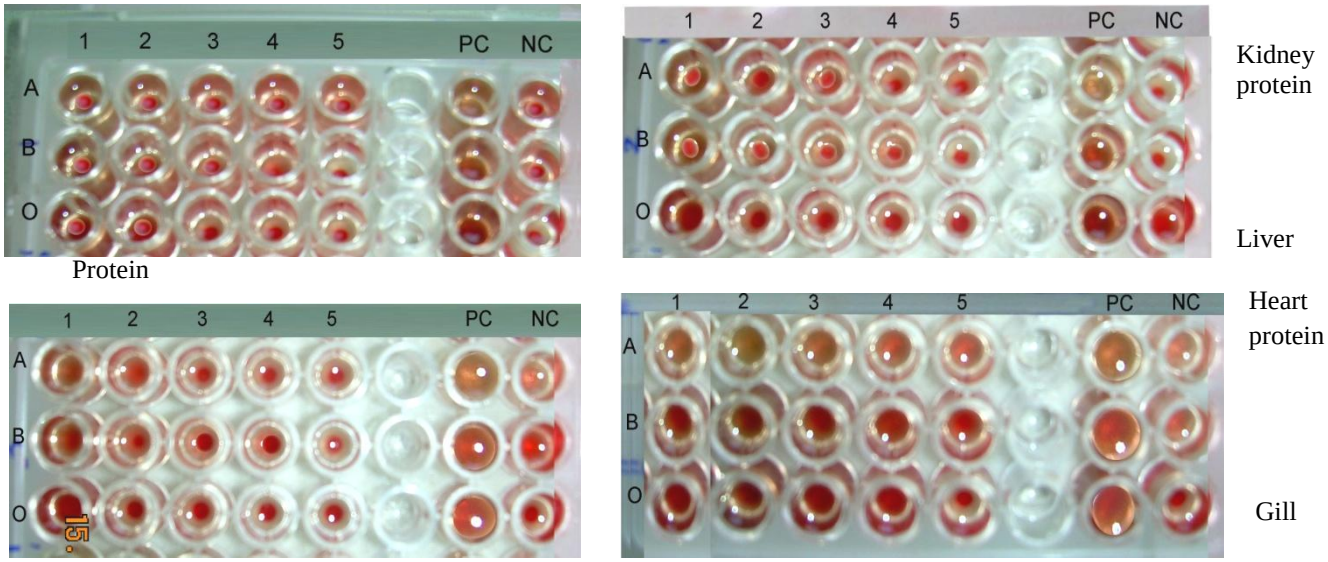


Figure 3. Haemolytic activities of kidney, liver, heart and gill proteins of *Labeo rohita* against ABO h RBCs



Note: The numbers (1-5) indicate the serial descending concentrations of proteins (500 -16 µg). PC & NC indicate the positive (100% haemolysis with Triton 100 X) and Negative (0% haemolysis without peptone or Triton 100 X) controls.

Minimum Inhibitory Concentration

The MICs of the tissue proteins against all the seven test organisms were determined and the results were shown in the Fig.2. All the four proteins have inhibited the growth of most of the organisms at the lowest concentration subjected i.e 16µg of protein. The growth of *K. pheumonia* was inhibited by somewhat higher concentrations (125µg) of kidney, liver and gill proteins and 500µg of heart proteins. Similarly the heart protein required to inhibit the growth of *S. typhimurium* also was found to be 500µg.

Haemolytic Activity

The partially purified proteins were also subjected to hemolytic assays with human 'A' 'B' O' erythrocytes in order to check their cytotoxicity to the human cells. The visual observation showed the button formation in all the wells of the microtitre plate except the positive control (100% haemolysis by triton 100X) for all the proteins. This result reveals that no protein has hemolytic effects on human 'A' 'B' 'O' erythrocytes (Fig. 3).

DISSCUSION

The antimicrobial proteins/peptides (AMPs) have been well documented as a part of innate immune system of plants and lower invertebrates to higher animals since long ago (Brogden, 2005). But the use of AMPs as a natural antibiotics to treat the infectious diseases has attracted the researches in recent years only because of their antiresistant nature. The AMPs are also believed not to cause side effects since they are animal orgins. Many investigators have studied the presence of AMPs in different type of cells and tissues of animal sources such as frog like amphibian, chicken like birds, sheep and primates like mammals including human beings (Zasloff, 2002; Ganz, 2003; Lehrer, 2004).

In this work the antibacterial proteins from kidney, liver, heart and Gill tissues of *Labeo rohita* were partially purified by acid extraction and by 30% ammonium sulphate solution. The proteins exhibited potential antibacterial activities on many organisms at lowest concentrations (16µg). It is in agreement with many earlier studies. The MIC range of TAP was observed to be 6-50 µg/ml for four bacterial strains (E.coli, K. pneumoniae, P. aeruginosa and S. aureus) and an yeast cell, Candida albicans (Diamond *et al*., 1991). The epididymis protein 2(Ep2) isoforms of human and rhesus monkey kill more than 99.9% of bacteria at 50-100µg/ml (Avellar *et al*., 2004). Most of the cathelicidin peptides rapidly kill a wide range of microorganisms at micro molar and sub micro molar concentration, with a broad spectrum (Gennaro and Zanetti, 2000 & Zanetti *et al*., 1995). Xiao *et al*., (2006) have reported that the synthesized chicken fowlicidins had potent antimicrobial activity in a salt independent manner with MIC of 0.4-2.0µg.

Some of the earlier investigated peptides had cytotoxic effects on eukaryotic cells and found not to be suitable for treatment. Hence, they require some modification in their solvent or sequence to prevent their hemolytic properties. For example, SMAP-29 and fowlicidins (-1 and -2) of sheep myeloid cells and chicken respectively displayed potent salt independent activities against a range of gram-negative and gram-positive bacteria including antibiotic resistant strains with hemolytic property. The hemolytic activity of the both fowlicidins and SMAP-29 was reduced by 2-4 fold in the presence of 10%PBS (Xiao *et al*., 2006). The Trp/Pro-rich antimicrobial peptides tritrpticin and indolicidin are relatively short peptides consisting of only 13 residues, and containing high fractions of tryptophan and proline. Although both peptides have broad spectrum of antimicrobial activities against bacteria and fungi, their relatively high toxicity toward eukaryotic cells prevent their usage as antibiotics (Yang *et al*., 2002 and Falla *et al*., 1996). Therefore much efforts has been taken in the

past decade to reduce cytotoxicity and to increase the cell selectivity of these two peptides (Subbalakshmi *et al*., 2000; Yang *et al*., 2002,2003 & 2006 and Ryge *et al*., 2004). Thus, the cytotoxic effects of many peptides prevent the development of peptide-based natural antibiotics unless some modifications done in their sequence even though they are potential to act against different pathogens. But, the proteins chemically purified from the organs of *Labeo rohita* may overcome this problem since they are exhibiting strong antibacterial effects without haemolytic effects.

According to Sundaramoorthy and Saravanan (2010), the TBS II Buffer extracts of goat and chicken heart tissues only displayed antibacterial activities against many clinical isolates such as *E. coli*, *S. aureus*, *Proteus*, *Mirrabilis*, *K.pneumoniae*, *P. aeruginosa* and *Salmonella* species whereas the same extracts of other organs (kidney, liver and lungs) did not show any bactericidal effects. It is notable that the proteins of all the organs of *Labeo rohita* partially and chemically purified in this study have significantly inhibited the growth of many bacterial strains including *S. aureus* which was not killed by the above mentioned buffer extracts of goat and chicken.

Thus antibacterial proteins isolated from the organs of *Labeo rohita* have advantages over other AMPs. Therfore, the identity of the antibacterial proteins will definitely provide a new insight for pharmaceutical applications.

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