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Antimicrobial Studies on Epidermal Mucus of Fish *Anabas testudineus*

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Abstract

The present investigation was conducted to find out the antimicrobial, hemolytic activity and protein content of fish epidermal mucus and their chemical constituents from Anabas testudineus. The in vitro hemolytic activity were analyzed and the antimicrobial activity against human pathogens (Bacteria) were determined by agar well diffusion methods. Epidermal mucus sample protein was analyzed by (Thin layer chromatography and SDS-PAGE). Totally thirteen human pathogens were tested against the fish mucus. Out of thirteen pathogens five pathogens have proved to be sensitive to the mucus. The average value of maximum zone of inhibition was observed against K. pneumonia (15.17±0.09mm) > P. vulgaris (13.2±0.17mm) > E. lentum (12.43±0.18mm) > MRSA (11.37±0.32mm) > S. aureus (10.5±0.11mm). The percentage of haemolysis for lyophilized sample shows more hemolytic activity. The amount of protein present in the mucus for Lyophilized sample: 3.61 g/dL, for Rotavac sample: 1.08 g/dL. The present inspection were revealed that positive progresses in the fish mucus extracts hostile to human pathogen (Bacteria) and the hemolytic activity, also the simple population of proteins (SDS PAGE) and the (TLC) reveals the presence of amino acids and peptides. Further efforts are required for the isolation of the active antimicrobial compounds in order to establish their possible applications.

Keywords: *Anabas testudineus, Fish Mucus, Antibacterial Activity, Hemolytic Activity, Protein Content*

1. Introduction

In the aquatic environment, fishes are in the constant interconnection with the wide range of pathogenic and non-pathogenic microorganisms and therefore possess complex defense mechanism which contribute to their survival (Balasubramanian, et al., 2011, Sangeetha Subramanian, et al., 2007, Nagashima, et al., 2001). The epidermal barriers is the first step of body defense, not only the fish species but also for all multi cellular organisms (Arslan Aydogdu et al., 2023, Kumari et al., 2011). Nowadays, fish mucus extract has been reported many biological functions such as Antibacterial, Antiviral, Antifungal, Anti parasitic and their potential use in human medicine and in fish farming (Abareethan, 2021, Tanaka et al., 2010, Ebran et al., 2000, Lemaitre et al., 1996, Fouz et al., 1990, Austin & MancIntosh, 1988, Ingram, 1980, Fletcher, 1978). Depending on the species, skin mucus varies considerably in viscosity, thickness and glycoprotein (mucin content) which also represents the major components of mucus (Dash *et al.,* 2018, Udhayakumar et al., 2012, Negus, 1963, Shepard, 1993).

Fish are highly conditional on their innate immunity for initial protection against the microbial takeover both during the primary stage of their life. When the acquired immunity is still idle, as well as during their later stages, as the acquired immunity has scare memory and very brief secondary responses (Uzma shabir et al., 2022, Magnadottir, 2006, Subramanian et al, 2007). Fish species need and well organized immune response to cope with conquering of pathogenic microorganisms (Weijun Leng et al., 2022). Identifying a novel antimicrobial compounds which is inherent to specific organ

and tissues of an organisms could be the probable alternative to combat the drug resistant bacterial pathogen (Pravin kumar N et al., 2012).

Hence, this present study was under taken in an attempt to conduct an inquiry into assess the natural immune components and the characterization of the antibacterial activity of the epidermal mucus of *Anabas testudineus*. The biological interface between fish and their aqueous environment consist of a mucus layer composed of biochemically deserve secretions from epidermal and epithelial cells (Pickering, 1974, Ellis, 1999). Particularly the antimicrobial agent appears to plays an important role in aquatic organisms including fishes. Which are always expressed to pathogenic microorganisms through the surrounding water (Subramaniyam Bragadeeswaran *et al.,* 2011, Agharid Al-Rasheed et al., 2018). Fishes have co-evolved under the selective pressure by also developing a complex network of defense mechanism such as the adaptive immune system, (Rocio Diaz-Puertas *et al.,* 2023, RathinamVennila et al., 2011, C.URAIB et al.,2011, Ellis, 2001). Skin mucus performs its role by a continuous production and slough off, preventing the pathogens attacks and containing several antimicrobial factors, such as proteins, lysozymes, immunoglobulin and lectins (Dash *et al* ., 2018, Brinchman,2016, Alvarez- Pellitero, 2018, Saurabh & Sahoo, 2008). Mucus is the slimy secretion consisting of mucins and combination of other substances such as inorganic salts, immunoglobulin and lipids suspended in water giving it characteristic lubricating properties (Pearson and Browunlee, 2005).

Due to the misuse of existing antimicrobial agents, the competition against infections caused by antibiotic resistant strains grows more difficult day by day. WHO predicts that by 2050 human deaths from antibiotic resistant infection will reach 10million (O'Ncill 2016, Anita Bhatnagar et al., 2023, Kuppulakshmi et al., 2008, Ong yeong et al., 2010). The epidermal layer of the fishes are contains specialized glandular cells that produce mucins and alarm substances (Smith 1992). These substances having potential of antimicrobial and noxious properties (Knouft *et al.,* 2003). The epidermal mucus samples from the fish were extracted with acidic, organic and aqueous solvents to identify potential of antimicrobial agents including basic peptides, secondary metabolites, aqueous and acid soluble compounds (Nathalie Ebran et al., 1998). The determinations of hemolytic activity will provide a good understanding of innate immune compounds in the fish mucus, which is essential in valuing the hemolytic mechanisms.

This study was conducted to estimate the presence of mucus proteins. Thin layer chromatography can be used to monitor the progress of a reactions, identify compounds present in a given mixture, and determine the purity of substances (Fair *et al*., 2008, Laurence, 2004). The detachment of macromolecules in an electric field is called electrophoresis. A very common method for separating protein by electrophoresis uses a discontinuous poly acrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to resolve the proteins (Shapiro AL 1967, Weber et al., 1969, Laemmli UK, 1970). SDS (also called lauryl sulfate) is an anionic detergent, meaning that when deliquescence its molecules have a net negative charge within wide p^H range (Schagger, 1987). The purity of protein samples can be evaluated and the progress of a fractionation or purification procedure can be followed (Wiltfang *et al.,* 1991). Lower percentage gels may cause the low molecular weight proteins to migrate with or in front of the dye front. High percentage gel may prevent the high molecular weight proteins from dissociation (Hames and Rickwood, 1990).

2. Material and Methods

Collection of mucus from fish

Specimens of *Anabus testudineus* of length 16cm±1cm and weight 72±1g were procured from the fish farm at kolathur, Tamilnadu. The collected fishes were acclimated to laboratory conditions in dichlorinated tap water for 15 days.

Preparation of skin mucus extraction

Sample A

Mucus collection was performed by using the method of (Ross et al., 2000) with slight modification. The fish were starved for 24 hours and the mucus was collected with a sub-lethal dose of 2-

phenoxyethanol. Individual anaesthetized fish were transformed into a polyethylene bags containing 10ml of 50mM Nacl. The mucus was immediately collected and transferred to 15ml sterile centrifuge tubes and placed on dry ice. The sample were centrifuged at 1500×g for 10min 4° C the supernatant obtained was aliquoted into 2ml centrifuge tube, freeze dried and stored at -80° C. A portion of mucus was lyophilized and suspended in phosphate buffer (PSB) P^H 7.4) at 1mg/ml concentration to give the aqueous extracts (Extract A).

Sample B

Mucus secretion were carefully scraped from dorsal side of the body using spatula by the method of (Jakowska, 1963). The mucus samples were mixed with 10% acetic acid in the ratio of 1:1 and placed in boiling water bath for 5min and it was cooled. Centrifuged at 18000 rpm for 35 min at 4° C. The resulting supernatant was evaporated overnight, by rotary evaporator (VC 100A Lark rotavapor at 30° C) with reduced pressure to give predominantly an aqueous suspension and stored at 4° C for further use.

Determination of protein

The estimation of protein was done by spectrometry according to the method of biuret. A calibration curve using BSA as standard. Reagent was measured against reagent blank at 540 nm.

SDS PAGE

Molecular standard were used to determine the molecular weight of mucus protein. The supernatant were analyzed by SDS-PAGE 10% topped by 5% stacking gel (Laemmli, 1970).

Thin layer chromatography

Aquatic extracts were applied thin layer chromatographic plate with a capillary tube and placed in a chamber containing methanol: chloroform (5:95) as developing agent using the method of (Jonathan et al., 2007). After development, compounds were visualized as purplish pink spots on spraying with 5% methanolic sulfuric acid as detecting agent followed by heating at 100° C till the spots were visible. Thin-layer chromatography profiling was done for the samples mucus extract in solvent system of butanol, acetic acid and water and in the proportion of 5:1:4. The plates when developed in the solvent system showing light pin spots, when the TLC plate is sprayed with 0.2% ninhydrin. The spots are shown on heating the plate at 100° C using the method of (Laurence et al., 2004)

Determination of hemolytic activity (in vitro hemolytic activity)

Freshly collected chick blood cells were taken and washed three times with 150mM Nacl by centrifugation method at 2500 rpm for 10 mins. The serum was removed and the cells were suspended in 100Mm sodium phosphate buffer. Three different concentration (5µl, 10µl and 15µl) of extracts were mixed with 200µ l of RBC solutions and the final reaction mixture volume was made up to 1ml by adding sodium phosphate buffer. The reaction mixture was then placed in water bath for 1hr at 37° C. After the incubation time the reaction was collected and the optical density was measured at 541nm (Amritha mukharjee and Rajasekaran, 2010).

Antimicrobial assay

Test pathogens

The total of 13 bacteria was taken from division of clinical microbiology Pondicherry center for biological sciences. The isolates are, *1. Pseudomonas aeruginosa, 2. Escherichia coli, 3. Vibrio cholera, 4. Proteus vulgaris, 5.Klebsiella pneumonia, 6.Salmonella typhi, 7.Eubacterium lentum, 8.Vibrio parahaemolyticus, 9.Enterococcus faecalis, 10. Bacillus subtils, 11.Enterobacter aeroges, 12.MRSA (Methiciline Resistant Staphylococcus auereus), 13.Staphylococcus Aureus*.

Preparation of test pathogen

The bacterial cultures were grown in Brain Heart infusion liquid medium at 37°C. After 12h of growth, each microorganism, at a concentration of 1×10^6 cells /mL is equivalent to 0.5 McFarland

standard was spread on the surface of Muller-Hinton agar plates. The dilution was made in sterile low glucose nutrient broth.

Antimicrobial activity (Agar well diffusion)

Test pathogens were spread on the test plates- Muller Hinton Agar (MHA) for bacteria and 6mm diameter well is made in the agar and load extract in 2mg/well concentration compared with streptomycin antibiotic (standard) loaded in the well in a concentration of 30 µg/well. DMSO (Dimethyl sulphoxide) used as Negative control. The test plates were incubated for 24h. The zone of inhibition (mm in diameter) were read and taken as the activity against the test Pathogen.

3. Results and Discussion

Determination of protein

The amount of protein present in the mucus sample of *Anabas testudineus* is: For Lyophilized sample: 3.61 g/dL. For Rotavac sample: 1.08 g/dL.

Thin layer chromatography

The plate with fraction developed, showing dark brownish spots indicating the presence of amino acids.

SDS PAGE

The fish skin mucus sample showed antimicrobial activity was subjected to SDS-PAGE. The stained gel revealed that the mucus contained a simple population of proteins. Only four clear bands in each of the mucus were detected in the gel. High intensity protein bands were detected in Lyophilized mucus sample.

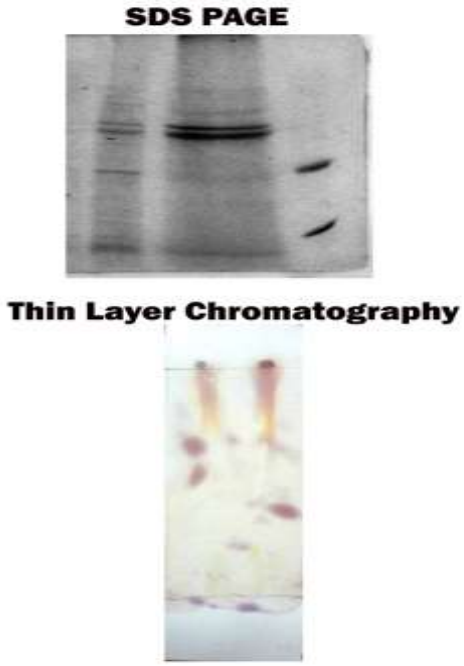

Figure 1: SDS PAGE & Thin Layer Chromatography

Antimicrobial activity

The comparative antibacterial activity of the mucus (lyophilized) from the *Anabas testudineus* using streptomycin as standard is given the table 1.

Table 1: Antibacterial Activity

Pathogen	Positive Control (Mm)	Extract (Mm in Diameter)
1. *K. pneumonia*	25.03±0.14	15.17±0.09
2. *P. vulgaris*	20.23±0.20	13.2±0.17
3. *E. lentum*	27.23±0.14	12.43±0.18
4. *MRSA*	-	11.37±0.32
5. *S. aureus*	20.4±0.23	10.5±0.11
6. *Enterobacter aerogens*	38.03±0.14	-
7. *P. aerginosa*	37.27±0.14	-
8. *V. cholerae*	30.33±0.20	-
9. *V. parahaemolyticus*	25.17±0.09	-
10. *S. typhi*	24.03±0.09	-
11. *E. coli*	18.07±0.14	-
12. *Bacillus subtils*	12.3±0.17	-
13. *Enterococcus feacalis*	10.13±0.14	-

The skin mucus sample collected from the *Anabas testudineus* shows a strong inhibition in the growth of tested bacteria. Species of gram positive bacteria (*E. lentum*, *Basillus subtils*, MRSA, *S. aerues*, *P. vulgaris*, *K. pneumoniae*). The average value of maximum zone of inhibition was observed against *K. pneumoniae* (15.17±0.09 mm), followed by *P. vulgaris* (13.2±0.17mm), *E. lentum* (12.43±0.18mm), MRSA (11.37±0.32mm), *S. aureus* (10.5±0.11mm). The mucus sample (Rotovac extract) was ineffective against the bacterial strains.

Haemolytic activity

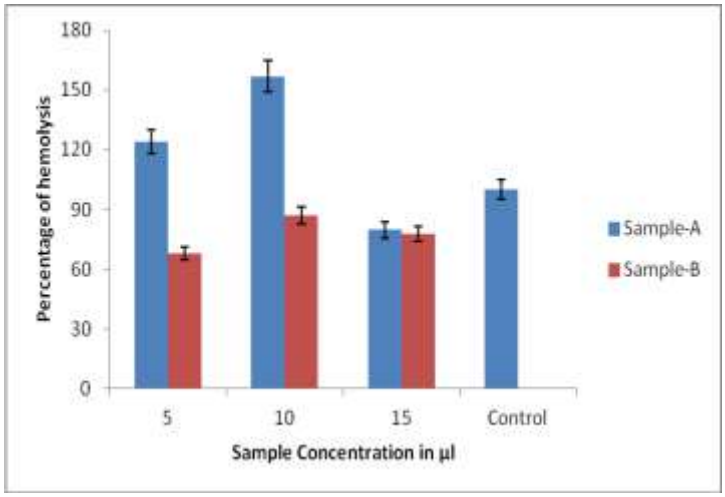

Figure 2: Hemolytic Activity

The fish mucus sample was subjected to haemolytic assay. The percentage of haemolysis for lyophilized sample (Sample A) shows more haemolytic activity. The result of hemolytic activity is revealed in figure 2.

The biological interface between fish and their aqueous environment consist of a mucus layer composed of biochemical diverse secretion from epidermal and epithelial cells (Pickering 1974, Ellis 1999). In the present study the mucus of *Anabas testudineus* shows the antimicrobial activity against pathogens. Fish secretes antibacterial proteins and peptides that are able to pass through the membrane of the target cells there by acting as a defensive barrier (Brinchmann, 2016). Generally fish mucus contains numerous antibacterial substances including antibacterial peptides, lysozymes and proteases that collectively plays a passive protective role (Hiwarale, et al., 2016, Ong yeong wei, et al., 2010). Many previous reports by numerous researchers also indicated towards the presence of potent bacteriacidal activity in epidermal mucus of wide range of fish species (Balasubramanian et al.,

2012, Nurtamin et al., 2016, Mahadevan et al., 2019). Among the fish by products fish, gills and blood is considered more valuable and has been reported that it contains several antimicrobial proteins (Praveen kumar, 2012).

Fish mucus is multifunctional material which plays a major role in communication, resistance to disease, respiration, ionic & osmotic regulation, feeding, nest building, reproduction and excretion (Ellis 1990, Subramanian Bragadeeswaran et al., 2011, Kerry Shephard,1994). Antimicrobial agents are normally present in the serum of fish (Kumari et al., 2011, Rathinam vennila et al., 2011, Pravin kumar et al., 2012, Venkatachalam Udhayakumar et al., 2012). Fish tissues and body fluids contain naturally occurring proteins or glycoproteins of non immunoglobulin nature that react with a diverse array of environmental antigen and many confer an undefined degree of natural immunity to fish (Uribe et al., 2011, Balasubramanian et al., 2012). Scale less fish species secreted more substantial amount of mucus than scaled fish (Dash et al., 2018).

Fish mucus is believed to play an important role in the prevention of colonization by parasites, bacteria and fungi and thus acts as a chemical defense barrier (Ramasamy Anbucheliyan et al., 2011). The protective layer of the surface mucus layer was previously investigated by (Fouz et al., 1990) who showed that the mucus of the turbot has an antibacterial actions against different pathogenic bacteria. Similarly (Austin and Mcinthosh 1988) demonstrated that antibacterial compounds existed on the surface of rainbow trout and predominantly inhibited *A hydrophila*. Antimicrobial compounds have been associated with and dispersed from the epithelial mucus-secreting cells of fishes (Cole et al., 1997).

Antibacterial results are similar to finding of earlier studies by various authors who observed that piscine mucus is a good antibacterial source of antimicrobial products which shows the strong antibacterial activity in several fishes (Balasubramanian, 2012, Prakash, et al., 2013, Nurtamin, et al., 2016, Kumari, et al., 2019). In the present study the skin mucus sample collected from the *Anabas testudineus* shows a strong inhibition in the growth of tested bacteria. Species of gram positive bacteria (*E. lentum, Bacillus subltils*, MRSA, *S. aerues, P. vulgaris, K. pneumoniae*). The average value of maximum zone of inhibition was observed against *K. pneumoniae* (15.17±0.09mm), followed by *P.vulgaris* (13.2±0.17mm), *E. lentum* (12.43±0.18mm), MRSA (11.37±0.32mm), *S. aureus* (10.5±0.11mm). The following 8 pathogens does not shown any activity against the mucus test sample except they have shown zone of inhibition for positive control only they are *Enterobacter aerogens* (38.03±0.14mm), *P. aerginosa* (37.27±0.14), *V. cholera* (30.33±0.20), *V. parahaemolyticus* (25.17±0.09), *S. typhi* (24.03±0.09), *E.coli* (18.07±0.14), *Bacillus subtils* (12.3±0.17), *Enterococcus feacalis* (10.13±0.14). The fish mucus sample does not show antibacterial activity on negative control.

In the earlier reported by (Bragadeeswaran and Thangarajan, 2011) has studied the specific activity of the fish species hemolytic and antibacterial studies on skin mucus. In the present study the mucus sample is tested against chicken blood sample showed a good haemolytic activity. The haemolytic activity is shown more for lyophilized sample (15µl) 156.85% is the highest percentage of haemolytic activity obtained. The mucus extract shows the hemolytic activity against chicken RBC as observed in the study and this reaction have the potential for application in medicine and the immune cell providing resistance and protecting against pathogens.

The amount of protein present in the mucus of *Anabas testudineus* is 3.61 g/dL for lyophilized sample. This indicates the samples have higher preservation of protein extraction. Thin-layer chromatography profiling showing pink spots indicating the presence of amino acids and peptides. The fish skin mucus sample showed antimicrobial activity was subjected to SDS-PAGE. The stained gel revealed that the mucus contained a sample population of proteins. SDS-PAGE has been previously used to determine the molecular weight of various AMPs isolated from the mucus of other fish species such as catfish, Atlantic halibut and common carp (Cole, et al., 1997, Park, et al., 1998, Birkemo, et al., 2003, Uzma shabir, et al., 2022). In this study there are only four clear bands in each of the mucus were detected in the gel. The increased amount of protein may be responsible for the high antibacterial peptides have been shown in the fish mucus. The appearance of protein in fish skin mucus contributes to its host shielding mechanism against bacterial infections.

4. Conclusion

Fish *Anabas testudineus* mucus showed antibacterial activity and haemolytic activity which is potentially useful for mankind. The fish mucus sample shown a remarkable antimicrobial activity and haemolytic activity against the 5 test pathogens out 13 pathogens have tested. The protein was present in a significant quantity in the lyophilized mucus sample without denaturing.

Conflict of Interest Statement

The authors state that they do not have any competing interests.

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