Bactericidal activities and biochemical analysis of skin mucus of Cyprinid Fish

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Abstract: This study reports the bactericidal activity of mucus extracts and biochemical characterization of skin mucus from five Cyprinids, including *Labeo rohita*, *Ctenopharyngodon Idella*, *Gibelion catla*, *Hypophthalmichthys molitrix* and *Cirrhinus mirigala* against ten different bacteria extracted from naturally infected fish. The bactericidal activity was measured based on the zone of inhibition (ZOI) and compared against Fosfomycin. Importantly, acidic mucus extracts from five fish species exhibited higher bactericidal activity than organic and aqueous extracts. The acidic skin mucus extracts of *C. idella*, *L. rohita,* and *G. catla* showed higher ZOI against *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. The minimum inhibitory concentration (MIC) of acidic mucus extracts from *C. Idella*, *L. rohita*, and *G. catla* was 16 μg/mL against *A. hydrophila*, *P. aeruginosa,* and *S. aureus*.

Further, biochemical characterization of mucus extracts showed that protein concentration was high in the acidic mucus extracts from *L. rohita*, *C. idella,* and *G. catla* compared to *H. molitrix* and *C. mirigala* followed by carbohydrate and lipid content. These findings suggest that skin mucus from cyprinids could be a potent source of innovative bactericidal components for fish and human-related treatments.

Keywords: Antimicrobial components; Bactericidal activity; Bacterial pathogens; Biochemical characterization; Cyprinids; Skin mucus

1. Introduction

Fish are in direct contact with water and are highly vulnerable to bacterial infections (Sudheesh et al., 2012). Several pathogenic bacteria have been observed in various fish species, with subsequent loss in their major tissues (Hamed et al., 2018). The bacterial diseases in fish include dropsy, epizootic ulcerative syndrome (EUS), swim bladder disease, scale loss, and tail and fin rot disease (Sudheesh et al., 2012). Contrarily, fish are equipped with skin mucus that provides defense against exogenous bacteria (Balasubramanian et al., 2012) with the help of immune-relevant components produced by goblet cells (Brinchmann, 2016). The skin mucus of fish serves as a protective layer between fish and the surrounding aquatic environment. It possesses essential biological and ecological functions(Reverter et al., 2018), such as osmoregulation and protection against abrasion, environmental toxins, heavy metals, and pathogens (Salinas, 2015). The skin mucus of fish is a dense fluid that changes composition as it moves over the surface and varies among fish species (Al-Arifa et al., 2013). The antibacterial factors present in fish skin mucus (Hedmon, 2018), can change against various physiological conditions in response to bacterial exposure (Reverter et al., 2018; Sridhar et al., 2021). Skin mucus from *C. mrigala* (Nigam et al., 2017), *C. catla*, *H. molitrix*, *C. idella,* and *L. rohita* showed inhibitory activity against pathogenic bacteria of fish (Balasubramanian et al., 2012). Hence skin mucus of fish act as a bactericidal; therefore, it needs detailed studies to be proved.

In Pakistan, freshwater fish are widely cultured on an industrial level in inland water (Shah et al., 2012). Fish face an outbreak of pathogenic bacteria in different culture systems (Mansoor et al., 2019), which renders their population and causes high economic loss. The pathogenic bacteria from infected fish may cause an increased risk of developing infections in humans after utilizing diseased fish (Kanwal et al., 2021). The bacterial affluence, antibacterial activity, and biochemical characterization of few fish skin mucus have been established (Nigam et al., 2017). Fish is a novel source these days for identifying and isolating novel bioactive compounds from its mucus, ethnic concerns about fish eating or its products apart from antibacterial, such as a good source of nutraceuticals and novel probiotic cultures (Ashraf et al., 2020). However, there is no information on fish skin mucus living in various climatic conditions in Pakistan. Thus, there is a probability of getting a diverse immune response and associated bactericidal factors which benefit them to live in unfavorable conditions. In order to characterize the bacteria from diseased fish, the current study reported bactericidal activity and biochemical characterization of skin mucus from *C. idella*, *L. rohita*, *H. molitrix*, *G. catla,* and *C. mrigala*.

2. Materials and Methods

2.1 Ethical approval

All methods used in this experiment were concented by the Research Ethical Committee of KUST1447, Kohat.

2.2 Isolation and characterization of bacteria

We collected fish from various fish farms in Khyber Pakhtunkhwa, Pakistan. We used the infected parts of the diseased fish for bacterial isolation, performed under aseptic conditions by serial dilution method. We determined the morphology and shape of bacterial colonies on the nutrient agar plates. Selective media such as MacConkey agar (MA) (Difco™, Becton, Dickinson and Company, NJ, USA), tryptic soy agar (TSA), eosin methylene blue (EMB), mannitol salt agar (MSA), and cetrimide agar were used for identification of bacterial species. Biochemical tests such as triple sugar iron (TSI), catalase, oxidase, motility indole urea (MIU), and sulfur indole motility (SIM) were also performed for the identification of bacteria as described earlier (Tonguthai et al., 1999). Luria Bertani (LB) broth was used to grown the pure culture of potential bacteria which were preserved in glycerol stock at -80 °C for further analysis.

Furthermore, DNA was extracted from purified bacterial samples, and universal primers (5′-ACGCGCGTGTGTAC-3′ Forward and 5′-CAGCCGCGGTMTA--3′ Reverse) were used for the amplification of bacterial DNA. PCR products were verified using 2% agarose gel electrophoresis in TBE buffer. Sequences acquired for gene 16sRNA were modified with Bio edit (created in MEGA X) and were submitted to GenBank. Sequences obtained were put in BLAST for searching the nearest neighbor species.

2.3 Skin mucus collection

Healthful alive fish (*C. idella, L. rohita*, *G. catla*, *C. mrigala* and *H. molitrix*) were kept in glass aquaria in the Laboratory of Fisheries and Aquaculture, Department of Zoology, Kohat University of Science and Technology (KUST), Kohat. Fish were kept starved for 24 hrs after seven days of acclimatization to maximize mucus secretion and avoid defecation during the mucus collection process. Fish was put on a sterile tray, washed with phosphate buffer saline, and gently scraped with a sterile slide from lateral sides. Skin mucus was collected from each 20 representative species and mixed. The collected mucus was put into falcon tubes (15mL) and lyophilized using Labconco's Freeze dryer and stored at -20 °C for Analysis of bactericidal activity (Nigam et al., 2017; Subramanian et al., 2008).

2.4 Preparations of skin mucus extracts

The extracted mucus from five fish was then partitioned into 3-portions, and isolated individually with acidic, aqueous, and organic (ethanol and methanol) solvents. The acidic mucus extracts were made with little modifications in Subramanian et al. (2008) protocols. Extracted mucus (15 mL) was homogenized with 80 mL of 5% (v/v) acetic acid and put in the water bath for 5 minutes. The acidic mucus was centrifuged at 18,000 rpm for 35 minutes at 4 °C after being properly vortexed and cooled to 4 °C. A reverse-phase Sep-Pak Vac 5 g C18 cartridge (125, 55-105 m; Waters Corporation, Milford, MA, USA) was used to collect and partially purify the supernatant. Before adding the supernatant, the cartridge was first activated with 15 mL of methanol and then equilibrated with 5 mL of 10% (v/v) acetic acid. Supernatant was placed into the cartridge, which was then washed once with 20 mL of an acetonitrile, water, and TFA mixture, persuaded by 5 mL of 0.1% (v/v) trifluoroacetic acid (TFA), before being eluted. The mucus samples were kept at -20°C, then submerged in distilled water and used for bactericidal activity.

Organic extracts (ethanol and methanol) were used to activate skin mucus. The extracted mucus (15mL) from all fish was immediately lyophilized and kept at -20 °C. Absolute ethanol was added to the dried mucus, then centrifuged at 11,000 rpm for thirty minutes at 4°C. Ethanolic extract was vortex thoroughly and was kept under liquid nitrogen for 24 hrs. Then, the ethanolic extract was re-suspended in 15 mL distilled water, followed by adding 5% (v/v) DMSO (dimethyl sulphoxide), thus finally used to evaluate the bactericidal activity. Methanol was added to dried mucus and centrifuged at 11,000 rpm for thirty minutes at 4°C. The methanolic extracts were mixed well and evaporated under liquid nitrogen for 24 hrs. To resuspend the dry pellet, 15 mL of distal water was added and extracted two more times with 50 mL of DCM (dichloromethane) then the mucus was analyzed for bactericidal activity. For aqueous extracts, 15 mL of the extracted mucus was re-suspended in 50 mM (w/v) ammonium bicarbonate under cold conditions and centrifuged at 10,000 rpm for ten minutes at 4 °C. The upper layer was amassed, immediately freeze-dried, and stored for further Analysis (Hellio et al., 2002).

2.5 Determination of the bactericidal activity of mucus

Bacterial species of 10^8 CFU/mL were cultured on petri plates containing 25 mL muller hinton agar (MHA). Different extracts of skin mucus were prepared with four concentrations (1-4 mg/mL). Each MHA petri plate was bored with three distinct wells and named AQ) aqueous, A) acidic, and O) organic with the positive control (Fosfomycin). Then, wells were punched aseptically with a sterile blue tip with a diameter of 6–8 mm, and 100 μL of each mucus extract per well was added. Alongside each mucus concentration, positive controls (Fosfomycin) and ethanol, methanol, acetic acid, and ammonium bicarbonate were used as negative control during bactericidal activity. Bactericidal activity was evaluated by quantifying the diameter of the ZOI produced across the well in a millimeter (mm) after 24 hrs.

2.6 Determination of minimum inhibitory concentration (MIC) for mucus extracts

The MIC is the least concentration of an analyzed bactericidal component that prevents the apparent growth of bacteria examined after 24 hrs incubation. Microdilution procedures was used to determine the MIC of acidic mucus extract using Muller Hinton Broth (MHB) in accordance with the Clinical and Laboratory Standards Institute (CLSI) (Wang et al., 2014) with minor changes. The acidic mucus extracts were 2-fold diluted, ranged in concentration from 256 to 2 μg/mL (100 μL mucus/per well). Evaluation of bacterial growth control (MHB + bacteria + mucus extract) was carried out immediately with one column each for negative control (MHB) and for the positive control (MHB + bacteria) used (Silveira et al., 2009). Microtiter plates were then incubated at 37 °C for 24 hrs. Each well absorbance was calculated by using a biometra microplate spectrophotometer reader at 630 nm.

2.7 Minimum bactericidal concentration (MBC)

MBC was performed according to (Pillai Jr, 2005)) with slight modification. MBC was performed after the MIC test by dispersing 5 μL of mucus sample from the microtiter plates on MHA plates that showed no apparent growth. MBC was recorded after 18–24 hrs incubation at the least concentration that produced 3-4 colonies, i.e., 99.9% of bacteria was inhibited

2.8 Biochemical characterization of fish skin mucus

Skin mucus extracts were prepared from the preserved mucus, thawed, and centrifuged at 5000 rpm for 5 minutes. For identification of biochemical constituents, 3 g of copper sulfate (CuSO4.5H2O), dissolve in 500 mL of 0.2 mol/liter sodium hydroxide, then 9 g of sodium potassium tartrate and 5 g of potassium iodide were added. Further, 1 mL of mucus of each species was procured in a distinct test tube, and added 1 mL of distilled water to a separate test tube that served as the blank. Furthermore, 3 mL of the biuret reagent was added to all the test tubes, involving the blank tube. The biuret reagent was mixed with mucus and warmed at 37 ºC for 10 minutes, and the absorbance was recorded through spectrophotometry at 595 nm against blank tubes. The standard curve was drawn, with the concentration of proteins along the X-axis and the absorbance along the Y-axis, to determine the protein concentration in each sample. The same process was adopted for protein analysis in the mucus of each species in triplicate.

The anthrone test estimated carbohydrate content. Briefly, 0.2g of anthrone was dispelled in 100 mL of diluted chilled sulfuric acid. 3 mL of anthrone reagent and 1 mL of mucus extract were incorporated in a test tube, and the mixture was then cooled in iced water. The reaction mixture was measured at 630 nm.

Lipid analysis was executed by a free fatty acid test. 5 g of mucus sample in a conical flask was mixed with 50 mL of ethanol. The burette was filled with a standardized solution of 0.1% NaOH, and a 2 mL phenolphthalein indicator was added. The solution was heated up to 40 °C, and the alkali solution (NaOH) was added to the mixture and gently shacked till a pale pink colour appeared that was shown the end point of titration, and absorbance was recorded.

2.9 Statistical analysis

The bactericidal activities of each fish skin mucus were analyzed in triplicate. Data were shown as mean ± SE from 3 replicate experiments. Significance was established using a one-way analysis of variance (ANOVA) where $P < 0.05$ were considered significant.

3. Results

3.1 Identification and characterization of isolated bacteria

This study observed the morphological and biochemical characteristics of different bacteria **(Table S1)** isolated from diseased fish (*G. catla*, *C. mrigala,* and *C. Idella*) **(Fig. S1A)**. Standard reference organisms were used to identified bacteria based on their cultural, morphological, and biochemical characteristics. Each bacterium produced round, rod-shaped, smooth, colorless, dew drop-like colonies on the petri dishes of nutrient agar and showed specific colour on respective media after incubation of 24 hrs **(Table S2) (Fig. S1B)**. The isolated bacteria were identified as *Edwardsiella* spp, *Aeromonas* spp, *Serratia* spp, *Enterobacter* spp, *Pseudomonas* spp, *Salmonella* spp, *Staphylococcus aureus, Escherichia coli*, *Klebsiella* spp, and *Bacillus* spp.

The ten identified bacterial species belonged to three families Enterobacteriaceae (*Staphylococcus aureus* (ON915526), *Salmonella enterica* (ON920836), *Enterobacter cloacae* (ON920869), *Escherichia coli* (ON935728), *Klebsiella pneumonia* (ON935750), *Bacillus wiedmannii* (ON920835), *Edwardsiella tarda* (ON935051), and *Serratia marcescens* (ON920834), Pseudomonadaceae (*Pseudomonas aeruginosa,* ON935772) and Aeromonadaceae (*Aeromonas hydrophila,* ON920871). Amplified PCR product of genomic DNA of ten species using 16S rRNA bacterial universal primers generated 714-1251 bp amplicons (**Fig. 1**). The 16S rRNA gene sequencing results of all isolated bacterial DNA revealed 96-99% similarity with reference reported sequences. The relationship among sequences of 16S rRNA gene was clustered to each other in the phylogenetic tree (**Fig. 2**). These clusters were also intensely upheld by their high bootstrap values.

3.2 Mucus secretion

Secretion of skin mucus was different in quantity and appearance among each species. Secreted mucus of *H. molitrix* showed less viscosity and soon became watery. While the mucus of *C. idella* and *L. rohita* was more viscous and secrete mucus in equal quantity in both winter and summer whereas *G. catla* secreted pale-yellow mucus with suffocating odor, and *C. mrigala* secreted less mucus as compared to the other species. Moreover, the secretion of skin mucus in all the species was more in winter than in the summer. Furthermore, the average length (cm) and weight (g) of all five selected fish were recorded as (*C. idella* 38±1.15cm; 949±1g, *L. rohita* 37±0.577cm; 799.6±0.577g, *G. catla* 34±0.57cm; 701±0.577g, *C. mirigala* 28.66±0.577cm; 499±1g, *H. molitrix* 30±1cm; 501±0.577g.

3.3 Bactericidal activity of mucus extracts

Among the four different extracts (aqueous, acidic, ethanol, and methanol), the acidic extracts have shown strong bactericidal activity than aqueous and organic mucus extracts (**Table 1-5**). All four concentrations (1-4 mg/mL) of fish species showed significant activity against identified bacterial species, which was comparable to the standard antibiotics. The activity of skin mucus extracts was increased with the increase in concentration, and high activity was recorded against *A. hydrophila, S. aureus* and *P. aeruginosa* at all concentrations. Variations in mean ± SE values of the ZOI of various mucus extracts used against identified bacteria were observed to be significantly ($P > 0.05$) higher against Fosfomycin. The acidic skin mucus extract from *L. rohita*, *C. idella*, and *G. catla* showed higher ZOI against *A. hydrophila* (44±1; 44±1; 42.3±2.51 mm respectively), *S. aureus* (45.33±1.15; 40.33±1; 40.6±1.52 mm respectively) and *P. aeruginosa* (44±1; 40.6±0.57; 44±1mm respectively) (**Table 1-3**). While *C. mrigala* and *H. molitrix* acidic extracts exhibited the least ZOI against *A. hydrophila* (29±2;35±1 mm respectively), *S. aureus* (31.6±1.52;32.66±0.577 mm respectively) and *P. aeruginosa* (39.6±1.52; 33.66±0.577 mm respectively) at 4 mg/mL concentration for each shown in (**Table 4-5**). Although aqueous and organic mucus extracts also showed potent bactericidal activity against identified bacteria, but the ZOI was not remarkably as high as in acidic mucus extract (**Table 1-5**). In the case of organic (ethanol) extracts of *L. rohita*, *C. idella* and *G. catla* skin mucus exhibited higher bactericidal activity against *A. hydrophila* (32±1; 34±1; 32±1 mm respectively), *S. aureus* (38±1; 38.66±1.52; 37±1mm respectively) and *P. aeruginosa* (37±1; 25±1; 38±1mm respectively) which were significantly (P>0.05) higher among all the identified bacterial strains compared with Fosfomycin (15.33±3.21; 14.66±1.15 mm) as well (**Table 1-3**). The aqueous extract of *L. rohita*, *C. idella* and *G. catla* also showed maximum inhibitory effect at 4 mg/mL against *A. hydrophila* (31±1; 32±1; 31±1mm respectively) *S. aureus* (26±1; 32.33±2.08; 25±1mm respectively) and *P. aeruginosa* (26±1; 32.6±1.52; 26±1 mm respectively) among all identified pathogenic bacteria (**Table 1-3**). Further, the aqueous extract of *C. mrigala* showed no remarkably bactericidal activity on initial concentration while with the increase of concentration it showed more activity (**Table 5**). However, skin mucus of *L. rohita* and *C. idella* was observed to be less active against *S. marcescens* and *K. pneumoniae*. and same is shown in **Table 1-2** and **Fig. 3,** for *G. catla*, the acidic and ethanolic mucus extracts for both *K. pneumonia* (29±1 mm; 21.33±3.21mm) and *S. marcescens* (39±1 mm: 26.33±0.57 mm) showed a maximum bactericidal effect. Photographic images of ZOIs of acidic, aqueous, and organic skin mucus extracts and one antibiotic against identified bacterial strains have been shown in **Fig. 3-4**, respectively.

3.4 MIC of potent acidic mucus extracts

The acidic extracts of five selected species were further explored for MIC activities against all identified pathogenic bacteria. The inhibitory concentration of acidic skin mucus extract was observed to differ for diverse tested bacterial species. MIC results were found to be different for acidic mucus extracts on different bacterial pathogens. *A. hydrophila*, *P. aeruginosa*, and *S. aureus* were found to be the most susceptible bacteria against acidic extracts of *L. rohita*, *G. catla*, and *C. Idella* at a concentration of 16 µg/mL. The same bacteria as *S. aureus* and *P. aeruginosa* were observed to be the most susceptible against skin mucus of *H. molitrix* and *C. mrigala* fish at the 32 µg/mL concentration. Among the skin mucus from five Cyprinid species, *L. rohita*, *C. Idella* and *G. catla* have the highest inhibitory activity as they inhibited 3 bacterial species at a concentration of 16 µg/mL compared to the activity of *H. molitrix* and *C. mrigala* **(Table S3)**. The acidic mucus extract of *C. Idella*, *L. rohita*, and *G. catla* exhibited the ability to kill the bacteria even at a lower concentration **(Fig. S2)**.

3.5 Biochemical characterization of mucus extracts

The change in colour from blue to purple or violet of different mucus extracts (acidic, organic, aqueous) showed the presence of proteins. Due to the presence of peptide bonds, the copper ions in the reagent undergo a charge reduction from +2 to +1, changing the colour from purple to blue. Among all the species, the acidic extract of *L. rohita, C. idella,* and *G. catla* has the highest protein content (303.6±1.52, 250±1.53, 240±1.53 μg/mL, respectively) compared to *C. mrigala* and *H. molitrix* (90±1.52: 100.79±1 μg/mL respectively). Similarly, in the case of carbohydrates content, the colour change in the skin mucus sample from pale yellow to blue dark green showed the presence of carbohydrates, where carbohydrate gets dehydrated when reacting with concentrated H2SO4 and forming a mixture. This mixture reacts with anthrone reagents to give a bluish-green colored complex. *L. rohita, C. idella,* and *G. catla* have shown the highest concentration of carbohydrates (100±1.52, 80±1.32, 67±1 μg/mL respectively) compared to *C. mrigala* and *H. molitrix* (50±1.52; 40.5±1.52 μg/mL respectively). Furthermore, the presence of lipids was confirmed by changing colour from dark pink to pale pink by adding a standardized alkali solution. The free fatty acids test showed that all the species have the least quantity of lipids compared to proteins and carbohydrates. The lipids content in *L. rohita, C. idella,* and *G. catla* (4.07±0.05, 3.1±1.52, 2.52±1 g/mL, respectively) were found more compared to *C. mrigala* and *H. molitrix* (1.57±1.53; 0.5±1 g/mL respectively) (**Table 6**).

4. Discussion

Due to increased knowledge of fish as a crucial source of protein for a growing population, there is a rising demand for seafood on a global scale. Wild fisheries are presently in a state of decrease because of over-fishing, changes in climate, pollution, and other influences. Though fish are cultivated on an industrial scale (Muddassir et al., 2019), which are facing a significant epidemic of bacterial infections with consequent economic losses (Ali et al., 2016; Shah et al., 2012). However, information regarding the pathogenic bacterial flora from fish in Pakistan is rare, and the fish industry is even in its early development (Ullah et al., 2022). Therefore, the current study aimed to study bacteria in naturally infected farmed fish. The bacterial species reported by this study from the freshwater fish of Pakistan were interesting addition to the previously reported bacterial species from the diseased fish of the surrounding world (Joseph et al., 2013).

Skin mucus of fish serves as a biological barricade between the fish body and the surrounding bacterial pathogens in the aquatic environment. Studies determined the defending functions of skin mucus and its components in different fish species (Dash et al., 2018; Leng et al., 2022; Subramanian et al., 2007; Zou & Secombes, 2011). This research study evaluated the bactericidal activity of fish skin mucus isolated with different solvents as aqueous, acidic, and organic (ethanolic and methanolic), and reported significantly varied results. Among all the 4 different extracts of skin mucus, acidic extracts revealed strong bactericidal activity against *A. hydrophila, S. marcescens, E. tarda, B. wiedmannii, K. pneumonia, E. coli, S. aureus, S. typhi* and *E. cloacae* (**Table 1-3**). Although aqueous and organic mucus extracts also showed bactericidal activity against various bacteria, the ZOI was not as high as in acidic mucus extract. Such significant bactericidal activity of acidic mucus extract was reported earlier (Hellio et al., 2002). These findings collectively show that the components in acidic extracts present in the fish skin mucus have imperative functions in host immunity in the aquatic environment against bacteria (Shapo et al., 2007). Organic extract of the skin mucus from all the fish species in this study exhibited bactericidal activity, however, less than acidic extract, which probably shows that the bactericidal components could either be less in number or not be much activity in the skin mucus isolated with organic and aqueous solvent compared to acidic extracts, which need further investigation. The efficacy of skin mucus extracted with acidic solvent against pathogenic bacteria was due to the high solubility of mucus proteins in acetic acid than organic solvents (Hancock & Sahl, 2006). The bactericidal activity of fish skin mucus isolated with an organic solvent may hint that fish skin mucus could be used against bacteria with an alternative solvent as such extracts are rich in several secondary metabolites. The positively charged protein components in the skin mucus are thought to counteract the negatively charged bacterial membrane and create holes in the membrane by accumulating bactericidal components (Subramanian et al., 2008). Further studies could better underpin the precise number and nature of immune factors in the fish skin mucus extracted with different solvents that undergo bactericidal activity.

In the current study, the bactericidal activity of aqueous extract of skin mucus was highly varied in terms of effectiveness among species and compared to antibiotics. This result was parallel with the previous studies, which reported the bactericidal activity of aqueous extract of various fish skin mucus (Gobinath & Ravichandran, 2011; Subramanian et al., 2007). The observed variations in bactericidal activity are thought to be due to the different compositions of skin mucus secreted by different Cyprinid species. The cells produced by the skin epidermal and epithelial vary among fish species and thus influence the composition of fish skin mucus (Subramanian et al., 2008). Our study indicates that bactericidal potency is present in the aqueous extract of skin mucus from different fish. Notably, the species-specific varied skin mucus may minimize the chance of bacterial resistance invading the fish.

The MIC of skin mucus extract of a few fish species against various pathogenic bacteria has been observed (Rao et al., 2015). In our results, MIC of the acidic mucus extract of *L. rohita, G. catla,* and *C. idella* was 16 μg/mL against *A. hydrophila, S. aureus, P. aeruginosa,* whereas those of *H. molitrix* and *C. mrigala* showed 32 μg/mL. Previously, the MIC of acidic extracts of *Tinca tinca* skin mucus was 60 μg/mL against *A. hydrophila*. In comparison, *Oncorhynchus mykiss* and *Cyprinus carpio* showed MIC against *S. aureus* at 50 μg/mL (Ebran et al., 1999). (Hellio et al., 2002) reported the MIC value in the range of 25–48 μg/mL of skin mucus of different fish species against various pathogenic bacteria. Generally, our results are according to the previous reports; however, the difference in the MIC value of skin mucus may be varied with fish species and bacterial diversity (Hancock & Sahl, 2006). The higher bactericidal activity (in terms of MIC) is due to the cationic peptides with greater isoelectric points are more soluble in acidic environments (Hancock & Lehrer, 1998; Ming et al., 2007). The skin mucus of fish varies greatly with physiological and ecological conditions, and the skin mucus-generating cells located in the skin epithelial layer also vary among the fish species (Kumari et al., 2019; Nigam et al., 2012). Even though fish skin secretes more mucus with different factors in the winter than in summer (Jung et al., 2012). Taken together, ecological factors such as dissolved oxygen, pH, temperature, and invading bacteria considerably affect the secretion of skin mucus in fish (Subramanian et al., 2008). Therefore, the MIC we determined, could be helpful in strategies of making skin mucus alternative to antibiotics and drugs against fish and human pathogenic bacteria.

It is recognized that the mucus of the skin acts as a mechanical shield at the border and adjacent pathogenic bacteria (Reverter et al., 2018). Fish skin mucus is the reservoir of antibacterial components that slough and trap bacteria due to their role in innate immunity

(Holm et al., 2015). The skin mucus from various fish such as *Channa striatus*, *Arius maculates,* and *Anguilla japonica* is proteinaceous (Manivasagan et al., 2009). The mucus of acidic extracts from different fish species was rich in proteins varying from 100.79 ± 0.03 to 305.00 ± 1.64 mg/mL when compared with other extracts of fish skin mucus (Kumari et al., 2019). The protein content in acidic extracts of skin mucus in our study varies from (90±1.52 to 303.6±1.52 μg/mL) of all the fish species. The relatively less content of proteins observed in our study could be due to the varied fish and climatic factors such as the pH of water, water quality, and the incidence of impurities. Although protein was a major component in the acidic extracts of skin mucus in all the fish of our study followed by carbohydrates and lipids. The findings of the current study are consistent with preceding work (Manivasagan et al., 2009) that found high protein content in the skin mucus of *Aulostomus maculates* and *Hypophthalmichthys nobilis* followed by carbohydrates and lipids. Further transcriptomic and proteomics-based studies could better establish the composition of fish skin mucus and the function of its potential immune components.

5. Conclusions

This pioneer report isolated and characterized the pathogenic bacteria from naturally infected farmed fish of Pakistan. The bactericidal activity of skin mucus from five fish species was established and the protein, carbohydrates, and lipid contents in the skin mucus from each species were measured. The information regarding the pathogenic bacteria will pave the way for the prevention of their possible transmission between the cultivable fish species in the studied region. The high bactericidal activity of the acidic skin mucus extracts of *G. catla*, *L. rohita,* and *C. idella* indicates the important bactericidal factors that can be used as resistant elements against bacteria. It may also hint that skin mucus can be used as an antibiotic with a lower challenge of antibiotic resistance and can be established as a cost-effective product.

Author Contributions: S.A, F.U.D, and W.U designed the research study; S.A and S.Z conducted the experimental work; S.A, F.U.D, and M.R analyzed the data; S. A., F.U.D, and M.N.K.K drafted and finalized the Manuscript. All authors have read and proofed the Manuscript.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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Table 1. Zone of inhibition (ZOI) shown by different extracts of skin mucus from *L. rohita* against different identified pathogenic bacteria

Concentration (mg/mL)	Mucus extract	Bacterial Strains										
		A. hydrophila	***E. tarda***	***S. aureus***	***E. coli***	***K. pneumonia***	***P. aeruginosa***	***B. wiedmannii***	***S. enterica***	***E. cloacae***	***S.* marcescens**	***P-Value***
1mg/mL	Aqueous	13.33±1.52	9.66±0.577	14±1	10.3±1.52	8.66±0.577	15.66±0.577	11±1	10.33±0.577	9±1	9.33±0.577	0.01
	Acidic	24.33±1.52	18±0.577	19±1	9±1	9.66±0.577	23.66±0.577	18.66±1.52	13.33±1.52	20±1	18.33±1.52	0.01
	Ethanol	15±1	10.3±1.52	17.66±1.5	10.66±1.52	10.33±1.15	16.66±1.52	9±1	14±2	14.33±2.08	14.33±2.08	0.05
	Methanol	14±1	11.3±1.15	14±1	13±2.64	12.66±2.51	8.6±2.64	9±1	8.66±1.52	9±1	8.66±1.52	0.05
2mg/mL	Aqueous	18.33±1.52	14±1	18.33±1.52	15±1	13±1	17±1	10±5	14±1	15.33±1.52	9±1	0.01
	Acidic	32.33±1.52	30.3±2.51	42.3±2.51	16.66±2.88	22.33±2.51	28.33±1.52	12.33±2.51	16.66±2.88	16±1	12.33±2.51	0.001
	Ethanol	30±1	21±1	25±1	14±1	21±1	26±1	20.33±1.52	23.66±1.52	22±1	23±1	0.01
	Methanol	11.66±1.52	29±1	23±1	7.66±1.52	15±1	16±1.73	19±1	24±1	18.66±1.52	14±1	0.01
3mg/mL	Aqueous	24±1	19±1	23.6±0.57	20±2	22.33±1.52	23±1	22.66±1.52	19±1	21.33±1	20±1	0.01
	Acidic	34±1	29±1	43.33±1.52	26.66±1.52	30.33±1.52	35±0.5	19.66±1.52	27±1	33.66±0.57	33±1	0.001
	Ethanol	34±1	26±1	27.33±1.52	19±1	25.66±0.57	34.66±0.577	23.66±1.52	23.33±1.52	21.33±0.57	20.33±1.52	0.01
	Methanol	28±1	20.66±1.15	27±1	11.66±1.52	11.66±1.52	29±1	19±1	27±1	26±1	18±1	0.01
4mg/mL	Aqueous	31±1	19±1	26±1	21±1	18.66±1.15	26±1	17.33±1.52	25±1	25±1	19±1	0.01
	Acidic	44±1	33±1	45.33±1.15	34.33±1.52	33±1	44±1	33±1	28±1	31±1	39±1	0.0001
	Ethanol	32±1	21.33±1.52	38±1	24.66±0.577	24±1	37±1	31.66±0.57	31±1	19±1	26.33±0.57	0.0001
	Methanol	31±1	31±1	35±1	19±1	14.66±1.52	28.66±1.52	22±1	19±1	18±1	19±1	0.0001
200μg	Fosfomycin	14.66±1.52	13±1	13±1	12±1	12.33±0.577	14.66±1.15	13.66±1.52	12.66±2.51	14.66±0.57	14.6±0.577	0.0002

Values are mean ± SE of mean. Statistical significance between different skin mucus extracts was determined using one way ANOVA (* $P < 0.05$).

Table 2. Zone of inhibition (ZOI) shown by different extracts of skin mucus from *C. idella* against different identified pathogenic bacteria

Concentration (mg/mL)	**Mucus extract**	**Bacterial Strains**										
		A. hydrophila	***E. tarda***	***S. aureus***	***E. coli***	***K. pneumonia***	***P. aeruginosa***	***B. wiedmannii***	***S. enterica***	***E. cloacae***	***S.* marcescens**	***P-Value***
1mg/mL	Aqueous	14.66±0.577	9±1	17.33±1.52	12.33±1.52	4±1	15±1	4.66±0.577	11.66±1.52	8±1	5±1	0.01
	Acidic	19±1	9.66±0.577	16.88±2.88	8.66±1.52	9.33±1.52	15±1	8±1	13.66±1.24	11±1	9±1	0.01
	Ethanol	10±1	6±1	14±2	7.33±2.08	9±1	13±1	3.33±0.577	9±1	12±1	11.33±1.52	0.05
	Methanol	9±1	4±1	8.66±1.52	5±1	5±1	15±1	6.66±1.52	4.33±0.577	12±1	15.66±1	0.01
2mg/mL	Aqueous	15±1	12.66±2	14±1	12±2.08	13±1	16±1	12±1	14±1	11±1	14±1	0.01
	Acidic	16±1	18.33±1.52	18±1	16±1.52	19±1	21.66±2.08	16±1	18.66±1.52	22±1	21±1	0.005
	Ethanol	12.33±1.52	11±1	18.66±1.52	11.66±0.577	10±1	14±1.73	11±1	11±1	10±1	12±1	0.05
	Methanol	15.66±2.51	14±1	21.33±0.577	13.33±1.5	13.33±0.577	17.33±1.52	10.33±4.6	12±2	16±1	15±1	0.05
3mg/mL	Aqueous	17±1	21±1	22.66±1.52	19±1	22.6±1.52	18.3±1.52	16±1	24±1	19±1	15±1	0.01
	Acidic	24±1	25±1	28±2	23±1	17.33±2.08	24±1	27.66±1.15	24.3±1.52	20±1	16.33±0.577	0.01
	Ethanol	25.66±1.52	18.6±1.52	24.6±0.57	20±1	19.33±0.577	14±1	21±1	21±1	25.6±1.52	29±1	0.05
	Methanol	28±1	15.3±1.52	22.6±3.78	18±1	15±1	17±1	15.6±1.52	11.6±1.52	8±1	8±2.64	0.01
4mg/mL	Aqueous	32±1	31±1	32.33±2.08	30±2.64	8.33±0.577	32.6±1.52	13±2	9.33±0.577	9±1	16±1	0.0001
	Acidic	42.3±2.51	38±1	40.6±1.52	37.3±1.52	13.33±1.52	40.6±0.57	17±1.73	29.33±2.08	28.33±1.52	13±1	0.0001
	Ethanol	34±1	29±1	38.66±1.52	24.6±1.52	18±1	25±1	12.66±1.5	18±2.64	19±1	13±1	0.0001
	Methanol	30.3±1.52	23.3±2.08	30±1	28.3±2.08	12±1	28±1.52	13.3±2.08	18±1	15±1	17.6±1.52	0.0001
200μg	Fosfomycin	14.66±2.88	14±1	12.6±0.577	14±1	13.6±2.08	12.66±1.15	12.33±0.57	13.3±0.577	14.6±0.577	13±2.64	0.02

Values are mean ± SE of mean. Statistical significance between different skin mucus extracts was determined using one ANOVA (* $P < 0.05$).

Table 3. Zone of inhibition (ZOI) shown by different extracts of skin mucus from *G. catla* against different identified pathogenic bacteria

Concentration (mg/mL)	Mucus extract	Bacterial Strains										
		A. hydrophila	*E. tarda*	*S. aureus*	*E. coli*	*K. pneumonia*	*P. aeruginosa*	*B. wiedmannii*	*S. enterica*	*E. cloacae*	*S.* **marcescens**	*P-Value*
1mg/mL	Aqueous	13±1	7.66±1.52	15±1	10.3±1.52	8.66±0.577	18±1	11±1	9±1	7.66±1.52	8±1	0.01
	Acidic	19±1	15.3±1.52	19.3±2.08	12±2	9.66±0.577	16±1	15.66±0.577	12.33±1.52	14.33±1.52	14±1	0.01
	Ethanol	15.33±1.52	10.33±1.52	16.66±1.52	10.6±2.08	12.33±2.51	17±2	8.66±1.52	13±1	13±1	13±1	0.05
	Methanol	15±1	9±1	14±1	13.6±2.08	12.33±2.08	16.33±2.08	9±1	7.66±1.52	8±1	8±1	0.05
2mg/mL	Aqueous	16.66±1.52	11±1	14.33±1.52	13.33±1.52	11.66±0.577	14.66±0.577	13±1	12±1	13±1	11±1	0.01
	Acidic	17±1	13±1	23.66±1.52	15.3±0.577	14.66±0.577	19.66±1.52	18±1	14.66±0.57	18.66±0.577	18±1	0.01
	Ethanol	14±1	13.66±1.52	15±1	12±1	13.66±0.577	16.33±1.15	12.33±1.52	13.66±1.52	12±1	11.66±0.577	0.05
	Methanol	17±1	14±1	18±1	15±1	15±1	16±1	9±1	10.66±1.52	10±1	10.66±1.52	0.01
3mg/mL	Aqueous	15.33±0.577	15±1	17.66±0.57	20±1	15.33±1.52	13±1	16±1	19±1	16.66±0.577	14±1	0.01
	Acidic	18±1	24±1	26.33±1.52	17.33±1.52	16.66±0.57	19.66±0.577	21.6 ±0.577	18±1	21.33±1.52	22.66±0.577	0.01
	Ethanol	14.33±1.52	18.33±1.52	17.66±0.577	20.66±0.577	18.33±0.577	14.66±0.577	23.66±1.52	23.33±1.52	21±2	21±1	0.05
	Methanol	15±1	20.66±1.15	27±1	11.66±1.52	11.66±1.52	19±1	19±1	27±1	26±1	18±1	0.01
4mg/mL	Aqueous	31±1	19±1	25±1	23±1	18.66±1.15	26±1	17.33±1.52	17±1	24±1	16.33±3.21	0.0001
	Acidic	44±1	33±1	40.33±1.15	34.33±1.52	29±1	44±1	29±1	28±1	31±1	39±1	0.0001
	Ethanol	32±1	19.66±4.16	37±1	24.66±0.577	21.33±3.21	38±1	31.66±0.57	31±1	19±1	26.33±0.57	0.01
	Methanol	31±1	25±1	35±1	16.33±3.21	14.66±1.52	28.66±1.52	22±1	17±2.64	18±1	19±1	0.01
200μg	Fosfomycin	15.33±3.21	12±1	14±1	13±2	14.33±0.577	14.66±1.15	12.66±1.52	14±1	14.66±0.577	12.66±0.577	0.02

Values are mean ± SE of mean. Statistical significance between different skin mucus extracts was determined using one ANOVA (* $p < 0.05$).

Table 4. Zone of inhibition (ZOI) shown by different extracts of skin mucus from *H. molitrix* against different identified pathogenic bacteria

Concentration (mg/mL)	**Mucus extract**	**Bacterial Strains**										
		A. hydrophila	***E. tarda***	***S. aureus***	***E. coli***	***K. pneumonia***	***P. aeruginosa***	***B. wiedmannii***	***S. enterica***	***E. cloacae***	***S. marcescens***	***P-Value***
1mg/mL	Aqueous	13.6±1.52	11±1	15.6±1.52	14.6±1.52	8±1	18±1	7±2	5±1	13±1	10±1	0.05
	Acidic	22.6±2.51	19±2	23.3±2.08	21±1	15±2	25.33±2.08	13.3±2.08	20±1	17±2	14±1	0.01
	Ethanol	18.6±1.52	13±1	18.6±1.52	13.6±1.54	11±1	18±1	9±1	16.6±1.52	14±1	10.3±1.52	0.05
	Methanol	17.33±2.51	11±1	14±1	11±1	7±2	17±1	7.3±2.51	15±1	12±1	9±1	0.01
2mg/mL	Aqueous	18±1	14±1	19±1	18.6±1.52	11±1	18.3±1.52	11.6±1.52	11±1	16±1	13±1	0.02
	Acidic	28±1	27±1	28.6±1.52	16.5±1	19.3±1.52	30.3±1.52	15.3±1.52	27±1	21±1	16±1	0.01
	Ethanol	19.3±1.52	16±1	22±1	17.6±1.52	14±1	22±1	12±1	18.3±1.52	17.6±1.52	14±1	0.005
	Methanol	19±1	14±1	22.3±1.52	18.6±1.52	11±1	20±1	12.6±1.52	18.6±1.52	14.3±1.52	12.6±1.52	0.05
3mg/mL	Aqueous	20.33±1.15	15.33±0.577	20.66±0.577	19.66±0.577	14.33±1.15	22.66±0.577	14.66±0.577	19±1	18.66±0.577	14.66±0.577	0.05
	Acidic	31.33±1.52	28.66±0.577	31±1	30.66±0.577	23±1	32.66±0.577	18.66±0.577	29.66±0.577	24.33±1.15	19±1	0.01
	Ethanol	22.66±1.52	18.66±0.577	24.66±0.577	20.66±0.577	15.66±0.577	25±1	14±1	24.66±0.577	21±1	16.66±0.577	0.01
	Methanol	11.66±1.52	14±1	24.33±0.577	19.66±0.577	13.66±0.577	10.66±0.577	15.66±0.577	21±1	17.66±0.577	15.66±0.577	0.05
4mg/mL	Aqueous	24±1	17.66±0.577	22.66±0.577	24.66±0.577	16.66±0.577	25±1	17.33±1.52	21.66±0.577	20.66±0.577	16.66±0.577	0.005
	Acidic	35±1	30.66±0.577	32.66±0.577	31.33±0.577	25.33±0.577	33.66±0.577	20.66±0.577	31.66±0.577	26.66±0.577	22.33±1.15	0.001
	Ethanol	25.66±0.577	20.66±0.577	25.66±0.577	22.66±0.577	17.66±0.577	25.66±0.577	16.66±0.577	24.66±0.577	23.66±0.577	17.66±0.577	0.001
	Methanol	22.66±1.15	16±1	25.66±0.577	21.66±0.577	15.66±0.577	22.66±0.577	18±1	22.66±0.577	19.66±0.577	16.66±0.577	0.005
200μg	Fosfomycin	14.33±0.577	13.66±0.577	13.66±0.577	14±1	13.66±0.577	12±1	13.33±1.15	12.33±0.577	14.33±0.577	12.33±0.577	0.05

Values are mean ± SE of mean. Statistical significance between different skin mucus extracts was determined using one ANOVA (* $P < 0.05$).

Table 5. Zone of inhibition (ZOI) shown by different extracts of skin mucus from *C. mrigala* against different identified pathogenic bacteria

Concentration (mg/mL)	**Mucus extract**	**Bacterial Strains**										
		A. hydrophila	***E. tarda***	***S. aureus***	***E. coli***	***K. pneumonia***	***P. aeruginosa***	***B. wiedmannii***	***S. enterica***	***E. cloacae***	***S.* marcescens**	***P-Value***
1mg/mL	Acidic	13.66±1.52	11.6±2.08	18.6±1.52	11±2.64	12.33±1.52	14.66±0.577	17±2	11±1	12.3±2.08	12.6±1.52	0.01
	Ethanol	21.33±1.52	5.33±1.52	20±1	19±1	11.6±1.52	20±2	11.6±1.52	18±2.64	15.3±2.51	9±1	0.05
	Methanol	15.33±1.52	5.33±1.52	15.66±1.52	13.3±1.52	9.33±2.08	17±2	8±1	14.3±2.08	5±1	7.3±1.52	0.05
2mg/mL	Acidic	18.33±1.52	17.6±1.52	26.3±1.52	16±1	17.3±1.52	24±2	17±2	16±1	16.3±1.52	17.6±1.52	0.005
	Ethanol	19±2	16±1	18.3±1.52	12.6±1.52	17±2	21±1	16.6±1.52	17.6±1.52	16±1	14±1	0.01
	Methanol	20.66±1.52	14.3±1.52	21.33±1.52	13.6±3.21	9±1	19.3±2.51	12±2	16±1	9±1	11±1	0.05
3mg/mL	Aqueous	19±1	13.66±1.52	17±1	16.66±1.52	16.3±1.52	20.6±1.52	15.6±1.52	16.6±1.52	12.6±2.51	16.6±1.52	0.02
	Acidic	29±1	24.3±1.52	28±1	25±1	27±1	30.6±1.52	26.3±2.51	27.6±1.52	24.6±2.51	24.3±2.51	0.01
	Ethanol	22.66±1.52	18.6±1.52	24±2	21±1	20±1	27.3±1.52	20±1	20±1	21.6±1.52	18.3±2.08	0.01
	Methanol	23.66±1.15	13±1	21±1	11.33±1.52	13.3±1.52	22±1	10.3±2.08	21.3±1.52	18.6±1.52	15±1	0.01
4mg/mL	Aqueous	26±1	16.3±1.52	24.6±1.52	20.3±1.52	18.3±2.08	28.3±1.52	18.3±2.51	20±2	18.3±2.08	23.3±1.52	0.001
	Acidic	29±2	26.6±1.52	31.6±1.52	25±1	26±1	39.6±1.52	27.3±2.51	25.6±1.52	24±2	28.6±2.08	0.0001
	Ethanol	23.66±1.52	22.6±1.52	27.3±1.52	21±2	17.3±1.52	28±1	13.6±1.52	25.3±1.52	23.6±1.52	18.6±1.52	0.005
	Methanol	23.33±2.52	17±1	20±1	24.6±1.52	17.3±1.52	24±2	21.3±1.52	22.3±1.52	21.3±1.52	17.6±1.52	0.001
200μg	Fosfomycin	14.66±1.52	13.6±0.57	15±2	14±1	14.6±2.08	13±1	12.6±1.52	12±2.64	14±1	14.6±1.52	0.05

Values are mean ± SE of mean. Statistical significance between different skin mucus extracts was determined using one ANOVA (* $P < 0.05$

Table 6. Biochemical analysis of skin mucus extracted with different solvents from five fish species observed in the study

Fish Names	**Mucus Extract**	**Protein conc. (μg/mL)**	**Carbohydrates conc. (μg/mL)**	**Lipids conc. (g/mL)**
L. rohita	Acidic	303.6±1.52	100±1.52	4.07±0.05
	Ethanol	190.5± 1.53	60.5±0.5	2.05±0.07
	Methanol	150± 2.5	50.5±1.5	2.7±1.5
	Aqueous	100±1.5	30.2±1.5	1.2±1.5
C. idella	Acidic	250±1.53	80±1.32	3.1±1.52
	Ethanol	150±0.5	60.2±2.5	2.0±0.5
	Methanol	140±1.5	40.5±0.5	1.5±0.5
	Aqueous	100±1.5	30.2±1.5	1.2±1.5
G. catla	Acidic	240±1.53	67±1	2.52±1
	Ethanol	154±1.52	40.5±1.2	1±0.5
	Methanol	130±0.5	25±1.5	1±0.5
	Aqueous	100±0.5	20.2±1.5	0.5±1.5
H. molitrix	Acidic	100.79±1	50±1.52	1.57±1.53
	Ethanol	40.2±1.5	20.5±1.52	0.007±1.52
	Methanol	40.2±1.5	20.5±1.52	0.005±1.5
	Aqueous	20.5±1.5	10±0.52	0.002±1.5
C. mrigala	Acidic	90±1.52	40.5±1.52	0.5±1
	Ethanol	50.3±0.5	30.2±0.5	0.002±0.5
	Methanol	40.5±1.5	25.2 ±1.5	0.002±0.5
	Aqueous	20.5±1.5	10±0.52	0.002±0.5

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