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Original Article

BIOCHEMICAL CHARACTERIZATION AND ANTIBACTERIAL PROPERTIES OF FISH SKIN MUCUS OF FRESH WATER FISH, *HYPOPHTHALMICHTHYS NOBILIS*

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ABSTRACT

Objective: The present study was undertaken to characterize the biochemical composition and antibacterial activity of skin mucus of fish *Hypophthalmichthys nobilis* against different human and fish pathogenic bacterial strains *viz. Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus, Bacillus cereus* and *Aeromonas hydrophilla.*

Methods: Skin mucus of fish *H. nobilis* was collected by skin scarping method. Antibacterial activity of mucus extract was carried out by agar well diffusion method and measured in terms of zone of inhibition(ZOI) in mm. Antibacterial activity of mucus extract was then compared with two antibiotic amikacin and chloramphenicol. Minimum inhibitory concentration (MIC) of skin mucus extract was also determined.

Results: The biochemical characterization of epidermal mucus extract revealed the presence of proteins as a major component (265±2.64 µg/ml) followed by carbohydrate content (63.66±0.88 µg/ml) and lipid content (0.0077±0.66 g/ml) respectively. Remarkable antimicrobial activity against all the selected microbial strains was observed. Zone of inhibition (ZOI) shown by crude mucus extract against all the bacterial strains was found to be significantly higher than higher than Chloramphenicol.

Conclusion: The present study opined that skin mucus of this fish can be used as potential antimicrobial components.

Keywords: *Hypophthalmichthys nobilis,* Microorganism, Fish skin mucus, Antibacterial activity, ZOI, MIC

INTRODUCTION

The worldwide emergence of *E. coli, K. pneumonia, Haemophilus* sp., *S. aureus* and many other ß-lactamase producers have become a major therapeutic problem. Hospitals worldwide have become literal breeding grounds for some of the most deadly bacteria. It is now estimated that half of *S. aureus* strains found at many medical institutions are resistant to antibiotics such as Methicillin [1]. Indiscriminate use of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms [2]. Even though pharmacological industries have produced the number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased because microorganisms are highly efficient at modifying or acquiring genes that code for the mechanism of multidrug resistance [3]. Compounding the problem of multidrug resistance, it is necessary to search for new antimicrobial agents to combat infections and overcome the problem of resistance and side effects of currently available antimicrobial drugs. Several attempts have been made for exploring new antimicrobial drugs from natural sources including plant and animal products. In modern society, zoo-therapy constitutes an important alternative among many other known therapies practiced worldwide. Zootherapy is the healing of diseases by use of therapeutics obtained or ultimately derived from animals [4].

Fishes are a diverse group of animals and comprise almost half the number of vertebrate species in existence today [5]. Approximately 20 million metric tons of fish by-products are discarded annually from the world fisheries [6]. Fish by-products are rich in potentially valuable proteins, minerals, enzymes, pigments or flavors. Fish mucus, a fish by-product, is a key component of fish innate immunity. It acts as innate defense barrier of fish skin which continuously gets replaced and helps to prevent stable colonization of majority of infectious microbes such as bacteria, fungus into the fish body [7]. Fish mucus is secreted by epidermal goblet cells and comprises of mucins and other substances such as inorganic salts, immunoglobulin, proteins and lipids suspended in water giving it characteristic lubricating properties [8]. The composition, viscosity, and rate of mucus secretion vary from species to species and have been observed to change in response to microbial exposure or to environmental fluctuation such as hyperosmolarity and pH [9]. Fish skin mucus has been reported to secrete many antibacterial peptides [10-11]. *Channa striatus* is endowed with wound healing, antinociceptive, platelet aggregation, anti-inflammatory as well as mild antifungal and antibacterial properties [12]. In addition to antimicrobial peptides, fish skin mucus also contains C-reactive protein, lysozymes, lectin, flavoenzyme, immunoglobins etc. which protects fishes against pathogenic microbes in their surroundings [13-14]. Antibacterial properties of crude skin mucus from many fishes have been demonstrated against several human and fish pathogenic bacteria by many workers [6, 15-16]. *H. nobilis* is omnivorous and feeds on larger phytoplankton mostly on algal blooms [17], thus this species lines with an environment harboring many infectious microbes. Thus, the present study was focused on analyzing the biochemical characterization and antimicrobial activity of skin mucus of *H. nobilis.*

MATERIALS AND METHODS

Fish collection and acclimatization

Live fish, *H. nobilis* irrespective of sex, weighing 800-900 grams were purchased from the nearby fish culture pond and maintained in F. R. P. tank (1000 L capacity) at Fish and Fisheries Laboratory, Department of Zoology, Kurukshetra University, Kurukshetra. Half of the water of the tank was changed on alternate days. Dissolved oxygen was maintained at a preferable level in the tank with the help of low-pressure aerators and pumps. The health of fishes was observed daily, and dead fish or fish with lesions (if any) was immediately removed. The fish were fed daily at 3% of body weight with commercial/formulated feed during the acclimatization period.

Fish skin mucus collection

The fish were acclimatized for seven days and kept starved for 24 h before mucus collection. A collection of mucus was done by 'skin-scraping' from the body of test subjects. No anesthesia was given prior to mucus collection. Mucus was taken from 15 fishes dorso–

laterally by using a sterile plastic spatula. Mucus scraped first was discarded to avoid any bacterial contamination. Collection of mucus from ventral region of the fish was avoided to prevent mixing of urinogenital excreta. Fish skin mucus was placed in vials and kept frozen at 0 °C until use to avoid bacterial growth and protein degradation.

Preparation of mucus extracts and biochemical characterization

Two mucus extracts viz. crude mucus extract and aqueous extract were prepare from the previously preserved mucus. For crude mucus extract skin mucus preserved from 15 fishes was thawed and centrifuged at 5000 r. p. m for 5 min. The supernatant was subjected to qualitative and quantitative assays to estimate the biochemical constituents. To prepare aqueous mucus extract, collected mucus was thoroughly mixed with equal quantity of sterilized physiological saline (0.85% NaCl) and centrifuged at 5000 r. p. m for 5 minutes. The supernatant was analyzed for biochemical constituents. Protein analysis was done by Biuret test [18] and Lowry assays [19]. Carbohydrate content was estimated by Anthrone test [20] and Phenol sulphuric acid reaction [21] and lipid analysis was performed by free fatty acid test [22] and folch method [23].

Test microorganisms-procurement and maintenance

Antibacterial activity of fish skin mucus extracts was tested for six human pathogenic bacteria *E. coli, K. pneumoniae, P. aeruginosa* (Gram-negative bacterial strains), *S. aureus, S. epidermidis and B. cereus* (Gram-positive bacterial strains) and a fish pathogenic bacteria *A. hydrophilla* (Gram-negative strain). The bacterial strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh through Department of Biotechnology and Department of Zoology, Kurukshetra University, Kurukshetra, India. All the bacterial strains were grown in nutrient broth (0.5% peptone, 0.5% NaCl, 0.3% beef extract, distilled water, pH adjusted to neutral (6.8) at 28 °C) under biomedical safety protocols and conditions. 10 ml of nutrient broth was poured in flask and one loop of target bacteria was added to the flask and incubated for 24 h at 37 °C in incubator.

Antibacterial assay

In vitro antibacterial evaluation of fish skin, mucus extracts were assayed by agar well diffusion method [24]. 100 µl culture of different bacterial strains was spread on different culture plates containing 15 ml of nutrient agar media [1.5% agar-agar, 0.5% peptone, 0.5% NaCl, 0.3% beef extract, distilled water, pH adjusted to neutral (6.8) at 28 °C] by using a sterile cotton swab. Wells were made with the help of cork borer on the agar nutrient media plates suitably spaced apart. 100 µl of both mucus extracts, crude and aqueous were loaded in wells on different plates. The plates were then incubated at 37 °C for 24 h. The antibacterial activity was assayed by measuring the diameter (mm) of the inhibition zone formed around the well [25]. Amikacin and Chloramphenicol drugs were used to compare the antibacterial effect of fish mucus extracts. NaCl was used as the negative control along with two antibiotics in the determination of antimicrobial activity of aqueous mucus extract. Experiments were conducted in triplicates to determine the reproducibility.

Minimum inhibitory concentration

MIC represents the lowest concentration of an antimicrobial substance that inhibits the growth of a microorganism. Agar plate dilution test [26] was performed to determine the MIC of crude skin mucus extract against all selected microbes. Desired concentrations of crude mucus extract were prepared by volume/volume dilution with distilled water and poured in different wells on nutrient agar plates. Plates were incubated at 37 °C for 24 h.

Statistical analysis

The data so obtained were pooled separately for each parameter and expressed throughout as means±SE Significant difference in antimicrobial activity of fish skin mucus of different fishes among groups was tested by Analysis of variance (ANOVA) Duncan's multiple range tests for the experiments. Statistical significance was settled at a probability value of P<0.05. All statistics were performed using SPSS Version 11.5 for Windows.

RESULTS

H. nobilis huge secrete amount of mucus which was viscous in nature. We collected 10-15 ml of mucus/day. In our study, we also noticed that amount of mucus secretion also vary according to the season. *H. nobilis* was reported to secrete more mucus in summer than in winter.

Biochemical characterization

The presence of proteins in fish skin mucus sample was confirmed by Biuret test. Change in the colour of skin mucus sample from blue to purple or violet indicated the presence of proteins. Similarly, colour change in skin mucus sample from light yellow to blue-green indicated the presence of carbohydrates in skin mucus of *H. nobilis*. Skin mucus sample of *H. nobilis* gave pink color solution after addition of dilute alkaline (0.1% NaOH) thus confirming the presence of free fatty acids in the sample.

The results for quantitative analysis of fish skin mucus have been presented in table 1.

Table 1: Concentration of biochemical constituents of skin mucus of *H. nobilis*

Parameters	Value
Protein (µg/ml)	265.00±2.64
Carbohydrate (µg/ml)	63.66±0.88
Lipids (g/ml)	0.0077±0.06

All values are mean±SE of mean, Value of n (No. of experiments) = 6

Table 2: Zone of inhibition (mm) shown by crude mucus extract of *H. nobilis* against different bacterial strains

Fish			
Microbial strains	**Crude mucus extract**	**Amikacin**	**Chloramphenicol**
K. pneumonia	23.58 ± 0.67^{Bd}	33.50 ± 0.458^{Aab}	17.33 ± 0.19^{Cd}
E. coli	32.66 ± 0.56^{Ba}	33.50 ± 0.56^{Aab}	25.66 ± 0.19^{Ca}
P. aeruginosa	27.62 ± 0.62^{Bc}	33.00 ± 0.40^{Abc}	18.33 ± 0.34^{Cd}
S. epidermidis	32.83 ± 0.49^{Aa}	32.00 ± 0.23^{Ac}	21.16 ± 1.29^{Bbc}
B. cereus	29.25 ± 0.57^{Bb}	34.50 ± 0.31^{Aa}	24.43 ± 0.31^{Ca}
S. aureus	26.33 ± 0.96^{Bc}	32.16 ± 0.34^{Abc}	22.16 ± 0.51^{Cb}
A. hydrophilla	25.93 ± 0.71^{Bc}	33.33 ± 0.19^{Aabc}	20.00 ± 0.16^{Cc}

ZOI also include well diameter, All values are mean±SE of mean, Means with different letters in upper case in the same row are significantly (P<0.05) different., Mean with different letters in lower case in the same column are significantly (P<0.05) different., (Data were analyzed by Duncan's Multiple Range test), Value of n (No. of experiments) = 6

Table 3: Zone of inhibition (mm) shown by aqueous mucus extract of *H. nobilis* against different bacterial strains

Fish			
Microbial strains	**Aqueous mucus**	**Amikacin**	**Chloramphenicol**
K. pneumonia	13.16±0.49^{Bbc}	26.36±0.93^{Aa}	10.00±00^{Cb}
E. coli	16.55±1.10^{Ba}	25.26±0.62^{Aab}	12.70±0.21^{Ca}
P. aeruginosa	12.73±0.51^{Bc}	25.86±1.38^{Aa}	08.73±0.50^{Cc}
S. epidermidis	16.71±1.04^{Ba}	23.11±1.10^{Aab}	10.10±0.33^{Cb}
B. cereus	15.85±0.94^{Bab}	22.66±0.95^{Aab}	12.13±0.07^{Ca}
S. aureus	11.58±0.50^{Bc}	23.03±1.23^{Aab}	10.06±0.03^{Bb}
A. hydrophilla	16.03±1.16^{Bab}	21.75±1.01^{Ab}	12.76±0.17^{Ca}

ZOI also include well diameter, All values are mean±SE of mean, Means with different letters in upper case in the same row are significantly (P<0.05) different. Mean with different letters in lower case in the same column are significantly (P<0.05) different, (Data were analyzed by Duncan's Multiple Range test), Value of n (No. of experiments) = 6

Table 4: MIC shown by skin mucus extract of *H. nobilis* against different bacterial strains

Microbial strains	***K. pneumonia***	***E. coli***	***P. aeruginosa***	***S. epidermidis***	***B. cereus***	***S. aureus***	***A. hydrophilla***
MIC (µl/ml)	25.00	25.00	50.00	50.00	25.00	50.00	50.00
ZOI (mm)	7.00±00	7.00±00	12.73±0.51	16.71±1.04	20.38±6.11	11.58±0.50	16.03±1.16

ZOI also include well diameter, All values of ZOI are mean±SE of mean, Value of n (No. of experiment) = 6 (for both MIC and ZOI)

Antibacterial assay

Effect of crude mucus extract and aqueous mucus extract of *H. nobilis* against microbial strains has been presented in table 2 and table 3 respectively. Both crude and aqueous fish skin mucus extracts exhibited the ZOI against all tested bacterial strains. Crude skin mucus extract exhibited maximum ZOI against *S. epidermidis* (32.83±0.49 mm) followed by *E. coli* (32.66±0.56 mm).

In *S. epidermidis,* ZOI was higher than both the antibiotics, amikacin (32.00±0.23 mm) and chloramphenicol (21.69±1.29 mm). Crude mucus extract showed significantly higher ZOI than chloramphenicol whereas it was insignificant when compared with amikacin (table 2). When the antibacterial activity of aqueous fish mucus extracts against selected bacterial strains was compared with amikacin and chloramphenicol, amikacin showed a significantly higher ZOI followed by fish mucus extract and chloramphenicol. Aqueous fish skin mucus extract showed maximum ZOI against *S. epidermidis* (16.71±1.04 mm) followed by *E. coli* (16.55±1.10 mm) and *A. hydrophilla* (16.03±0.16 mm). No ZOI was shown by negative control (NaCl).

In the case of MIC assay, inhibitory concentration of mucus extract was found to vary for different microbial strains tested. MIC of crude mucus extract of *H. nobilis* was found in the range of 25 µl/ml to 50 µl/ml (table 4).

DISCUSSION

Fish skin mucus acts as the first line of defense against microbes [11, 27-28]. Negus (1963) reported that scaleless fishes produce a higher amount of epidermal mucus than fish with scale [29]. Although bighead is scaly fish, it also secretes a large amount of mucus. The quantity and quality of mucus have been reported to differ according to the season, environmental conditions such as pH, handling stress and age of fish [30-31] which also supports our findings that amount of mucus secretion was more in summers as compared to winters. All these factors play an important role in the susceptibility of a fish to infection [9, 13].

Crude mucus extract of *H. nobilis* is constituted of protein as a major component followed by carbohydrate and lipids. Manivasagan *et al.* (2009) investigated that soluble gel of *A. maculates* was having 12.64 µg/g of protein content,0.08 µg/g of carbohydrate content and 0.005 µg/g of lipid content[32] which also supports our results. Wei *et al.* (2010) also reported protein content in both crude and aqueous mucus extract of *Channa straitus* [6]. Dhotre *et al.* (2013) also characterized the biochemical composition of freshwater fishes viz. *Channa punctatus, Channa gachua, C. carpio and A. dussmieri* [33] and found similar results. Similarly, protein has been reported as a major component of fish skin mucus of six freshwater fishes viz. *Clarias gariepinus, Channa micropeletes, C. straitus, Oreochromis niloticus* and *Hemibagrus nemurus* [34]. The presence of protein content was also investigated in the epidermal mucus of Gaint snakehead, striped snakehead, *Tilapia mossambicus* and bagrid catfish [35]. Our results also go in agreement with the above studies. Review of the literature reveals that high amount of protein may be responsible for antibacterial activity shown by fish skin mucus [6, 32, 34-38]. Over the past few years, many antibacterial peptides have been isolated from different a fish which provides a non-specific innate immune system to fishes against various pathogen and help fishes to survive in adverse conditions [36, 38-40].

Crude mucus extract of *H. nobilis* exhibited strong antibacterial activity against all selected microbes. The Strong antibacterial activity of crude fish skin mucus extract has also been observed in other similar studies [15, 36, 41-42]. Wei *et al.* (2010) observed that both crude mucus extract and aqueous mucus extract of *C. straitus* showed inhibitory effect against fish pathogenic bacteria *A. hydrophilla* (8 mm) and no inhibitory effect against human pathogenic bacteria *E. coli* and *K. pneumonia* [6] whereas crude and aqueous mucus extract of *H. nobilis* showed strong antibacterial activity against both fish and human pathogenic bacteria. Bragadeeswaran and Thangraj (2011) noticed that crude mucus extract of eel fish show a strong inhibitory effect against *E. coli*, *P. aeruginosa* and *S. aureus* and no activity was observed against *K. pneumonia.* In the same study, they reported that aqueous mucus extract was not effective against *P. aeruginosa* [43]. However, crude mucus, as well as aqueous mucus extract of H. nobilis, exhibited antibacterial activity against all the four bacteria tested by Bragadeeswaran and Thangraj (2011). Loganathan *et al.* (2013) reported the inhibitory effect of crude mucus extract of *C. straitus* against *E. coli, S. aureus and Aermonas* sp.[44]. Our findings on crude mucus extract are in the agreement with above study. Mucus extract of *C. gaucha, C. punctataus, C. carpio* and *A. dussumieri* showed no ZOI against *K. pneumonia* [33]. Rao *et al.* (2015), did not notice the inhibitory effect of crude and aqueous mucus extract of *C. micropeltes, C. straitus, Chrysichtys nigrodigitatus* and *T. mossambicus* against *E. coli*. However, crude and aqueous mucus extract of *H. nobilis* exhibited strong antibacterial activity against *E. coli* as well as *K. pneumonia* in contrary [33, 35]. Aqueous mucus extract of *H. nobilis* also exhibited strong antibacterial activity against all pathogenic bacteria taken under study but comparatively lesser than antibacterial activity shown by crude mucus extract. Strong inhibitory effect of aqueous mucus extract shown by a variety of fishes *Arius caelatus*, *A. maculates*, *C. striatus, Clarias batrachus, Cynoglossus arel, Hertropneustes fossilis and Mystus gulio* [43, 45-48] support our findings on aqueous mucus extract. Subramanian *et al.* (2007) also reported the presence of antimicrobial compounds in aqueous mucus extract [38]. But in their further studies no antibacterial activity was observed in aqueous mucus extract of

wider range of fish species including Arctic char (*Salvelinus alpinus*) brook trout (*Salvelinus fontinalis*), Koi carp (*C. carpio*), striped bass (*Morone saxatalis*), haddock fish (*Melanogrammus aeglefinus*) and hagfish (*Myxine glutinosa*)[11]. Strong antibacterial activity exhibited by aqueous mucus extract of two indigenous fish (*Catla catla* and *Labeo rohita*) and two exotic fishes (*Hypophthalmichthys molitrix* and *Ctenopharyngodon idella*) [49] which also supports our findings. On comparing the results of Balasubramanian *et al.* (2012) with our study, *H. molitrix* was found to show higher antibacterial activity than *H. Nobilis*. Kumari *et al.* (2011) [16] reported antibacterial activity of aqueous mucus extract *Rita rita* and *Channa punctatus* against *S. arueus* (9.75±1.70 mm) but at the same time, no antibacterial activity was reported against *E. coli and P. aeruginosa*. However, our results showed that aqueous mucus extract of *H. nobilis* exhibit maximum antibacterial activity against *E. coli* (16.55±1.10 mm) followed by *P. aeruginosa* (12.73±0.51 mm) and minimum against *S. aureus*. (2008) [15] also studied the inhibition effect of aqueous mucus extract of *Channa punctatus* and *Cirrhinus mrigala* against ten pathogenic strains out of which 4 bacterial strains viz. *E. coli*, *K. pneumonia, P. aureginosa* and *S. aureus* are common with the present study. Our findings are in agreement with Kuppulakshmi *et al.* (2008) [15].

However, contradictory to our result no antibacterial activity was observed in aqueous mucus extract of 13 fish species [10]. Our observation on aqueous mucus extract also supports the reports on the antimicrobial nature of hydrolytic enzymes such as lysoymes, cathepsin B, trypsin-like proteases in fish mucus [11, 50-52]. Fish mucus extracts of *H. nobilis* were found to show strong inhibition effect against all the microbial strains taken under study. Thus, suggesting the presence of one or more antibacterial components in fish skin mucus of *H. nobilis*. Paradaxin pore forming a peptide, from Moses fish *Pardachius marmoratus* [41] and pleurocidin in skin secretion of winter flounder [36] have been isolated. Ebran *et al.* (1999) also reported pore forming properties of protein extracted from fish epidermal mucus [53]. The action of these antibacterial peptides is non-specific and rapid; they kill bacteria by a pore formation in cell membranes followed by disruption and solubilization [53]. Thus, we may assume that strong antimicrobial activity of epidermal mucus extracts of *H. nobilis* against microbial strains may be due to pore formation ability of their antibacterial peptides in target cell membrane.

MIC assay was carried out on mucus extracts of some fishes such as *C. statius, Desyatis sephen* and *Himantura gerradi* against many human and fish pathogenic bacterial strains [6-7]. Rao *et al.* (2015) reported the MIC value of Gaint snakehead, striped snakehead, tilapia and bagrid catfish (*C. nigrodigitatus*) against different pathogen ranged from 11.96µg/ml to 31.91 µg/ml. The MIC values reported in these works was not similar to those obtained in our study. In our study the minimum concentration of 50 µl/ml of skin mucus extract of *H. nobilis* was found to inhibit the growth of human pathogenic bacteria *S. epidermidis, S. areus, P. aeruginosa* and fish pathogen, *A. hydrophilla.* The minimum concentration of 25 µl/ml was adequate to inhibit the growth of *K. pneumonia*, *B. cereus* and *E. coli*. Same fish or different fishes exhibited different antibacterial activity against different or same bacterial strains. This may be due to difference in their age, geological and physiological conditions. Thus, skin mucus extract of *H. nobilis* needs to be characterized further, and can be explored as a potent antimicrobial against infectious bacteria.

CONCLUSION

The present findings suggest that epidermal mucus of *H. nobilis* is a good source of antimicrobial compounds. This antimicrobial activity might be due to antimicrobial proteins present in epidermal mucus as protein was found to be the major component of mucus. The epidermal mucus extracts of *H. nobilis* showed a different zone of inhibition against different bacterial strains. Thus, indicating antimicrobial activity of skin mucus of *H. nobilis*. Further, a detailed investigation is required for purification and characterization of specific antimicrobial components of epidermal mucus so that it may be utilized as potent anti microbe.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper

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