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Note

Antibacterial activity of epidermal mucus extracts of three freshwater air-breathing fish species against human pathogenic bacteria

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ABSTRACT

Mucus layer from epidermal secretions of the fish act as a first line of defense between fish and pathogens in their environment. Fish skin mucus has been reported to prevent colonisation of pathogenic bacteria. The objective of the present study was to explore the antibacterial activity of epidermal mucus extracts from three freshwater air-breathing fish species (*Clarias gariepinus, Heteropneustis fossilis* and *Channa punctatus*) against human bacterial pathogens. The crude, acidic and organic extracts of skin mucus were prepared and tested for antibacterial activity by disc diffusion method against three Gram positive (*Enterococcus faecalis, Staphylococcus aureus* and *Streptococcus pneumoniae*) and 5 Gram negative (*Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Vibrio inaba* and *Pseudomonas aeruginosa*) bacteria. Out of 72 tests performed (nine types of mucus extracts against eight different bacterial strains), 59 tests showed antibacterial activity. The order of the level of antibacterial activity of the mucus of the three fish species observed in the present study was, *C. gariepinus* > *C. punctatus* > *H. fossilis* and the acidic extract of *C. gariepinus* showed the maximum antibacterial activity against Gram negative bacteria.

Keywords: Acidic extract, Antibacterial activity, Crude extract, Freshwater fish, Mucus, Organic extract

Infectious diseases caused by bacteria pose important health issue currently (World Resource Institute, 2000) because of the development of multi-resistance in response to indiscriminate use of antibiotics. In spite of the vast development of chemotherapeutic techniques, the search for new antibacterial agents from natural sources is in high demand to combat the problem of drug resistance and side effects of the currently available antibacterial drugs. Attempts are being made for the development of antibacterial drugs from natural sources particularly derived from the animal source. Among animals, fishes constitute a major resource for a variety of bioactive compounds. Fishes are evolved to thrive in the environment which is considered to be rich in micro-organisms. The epidermal mucus in fishes acts as the first line of defence against invasion by microbes (Pinky *et al.,* 2007; Yashpal *et al.,* 2008; Kumari *et al.,* 2009). The epidermal goblet cells of fish skin secrete mucus which is comprised of gel-forming macromolecules like mucins and other substances such as glycoproteins, immunoglobulins and lipids (Negus, 1963; Shephard, 1993). Several attempts have been made to study the role of fish mucus in the prevention of colonisation of bacteria, parasites and fungi (Lemaitre *et al.,* 1996; Ebran *et al.,* 2000). Antibacterial activity of fish mucus was initially determined by Austin and McIntosh (1998) in rainbow trout *(Oncorhynchus mykiss)*. The removal of epidermal mucus resulted in increased susceptibility to bacterial infection in *Cyprinus carpio* (Lemaitre *et al.,* 1996). A variety of biologically active compounds like immunoglobulins, antimicrobial peptides, proteolytic enzymes, lysozymes, C-reactive protein, lectins and flavoenzymes of fish mucus provide immediate protection to fish against pathogens (Villarroel *et al.,* 2007; Kitani *et al.,* 2008). The antibacterial property of fish mucus differs from species to species and is specific against certain bacteria (Noya *et al.,* 1995). The present study was planned to investigate antibacterial activity of skin mucus of three air-breathing freshwater fishes *viz*., *Clarias gariepinus* (CG)*, Heteropneustes fossilis* (HF) and *Channa punctatus* (CP) using crude extracts (CEs), acidic extracts (AEs) and organic extracts (OEs) against selected human bacterial pathogens.

Individuals *C. gariepinus* (CG)*, H. fossilis* (HF) and *C. punctatus* (CP) were collected from river Gomti with the help of local fisherman. The fishes were acclimatised in laboratory conditions for 7 days and fed with commercial fish feed once a day. The health of all fish was monitored and injured/dead fish were removed from the aquarium.

Fishes were starved for 24 h and the mucus was scraped gently from the dorsal surface of the fish with the help of sterile spatula, avoiding contamination from sperm and intestinal excreta. The mucus collected from 9 individuals of each fish species was pooled separately representing one mucus sample from each selected species. A total of 62, 31 and 139 ml of mucus were collected from CG, HF and CP respectively. The mucus samples were centrifuged at 5000 rpm for 10 min at 4^0C. The supernatant was collected and stored in sterile vials at 0^0C to prevent degradation of protein and bacterial growth.

The preserved mucus was thawed and divided into three parts. One part of the mucus was used as CE. Second and third parts were utilised for the preparation of AE and OE respectively. For preparation of AE, 15 ml mucus was mixed with 15 ml of 10% (v/v) acetic acid and the mixture was kept for 5 min in boiling water bath, ice cooled, homogenised and centrifuged at 8000 rpm for 35 min at 4^0C. The supernatant was collected and filtered using 0.22 µm syringe filter. The filtrate was used as AE for evaluating antibacterial activity (Subramanian *et al.,* 2008).

Mucus samples were lyophilised and dissolved in 95% ethanol and the pellet was extracted (1 mg ml^{-1}) after centrifugation (7000 rpm for 30 min at 4^0C) of the solution, which was evaporated under vacuum below 40^0C. The extracts were dissolved in distilled water and partitioned with dichloromethane (DCM). The DCM (organic) phase was collected, dried and suspended in 5% (v/v) dimethyl sulphoxide (DMSO) (Hellio *et al.,* 2002). Antibacterial activity of the OEs of mucus of the three species of fish was evaluated.

Screening of skin mucus extracts of three species of freshwater fish for the antibacterial activity was carried out against three Gram positive (*Enterococcus faecalis, Staphylococcus aureus, Streptococcus pneumoniae)* and 5 Gram negative (*Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Vibrio inaba, Pseudomonas aeruginosa*) human pathogenic bacteria. All bacterial strains for the present study were procured from the Department of Microbiology, King George Medical University, Lucknow, India and were cultured at 37^0C for 24 h in Muller Hinton Agar (MHA, Himedia) except *S. pneumoniae* which was grown in 5% sheep blood agar (Biomerieux). For antibacterial testing, 3-5 colonies of each test strain of bacteria were mixed with 1.5 ml of normal saline and density of the suspension was adjusted to 0.5 McFarland units (10^8 CFU ml^{-1}), using density check meter (densiCHEKplus Standards Kit- Biomerieux). Sterile filter paper discs of 6 mm dia (Whatman filter paper no.1) were impregnated on the agar plates seeded with microorganisms and 10 µl of the extracts were loaded on the discs. The plates were incubated at 37^0C for 24 h. Standard antibiotics discs of Linezolid (Himedia) and Imipenem (Himedia) were used as positive controls for Gram positive and Gram negative bacteria respectively. Acetic acid (10% v/v), ethanol, DCM and DMSO (5%) which were used for extraction, acted as negative controls. All tests were performed in triplicates. Antibacterial activity was estimated in terms of diameters of zones of inhibition (ZOI) surrounding the discs.

Statistical analyses of the data on antibacterial activity of different mucus exracts against different bacterial strains were done using one-way ANOVA followed by *post hoc* Tukey's test. All statistical analyses were carried out using Graph Pad Prism software (version 5.01).

The details of the results of the antibacterial activity of the mucus extracts from three fish species are given in Table 1. Out of 72 tests performed, 59 showed antibacterial activity at 10 µl of the mucus extract (Table 1). Maximum ZOI was observed for AE of CG and HF against *K. pneumoniae* (26.4 mm), *V. inaba* (24.4 mm) respectively and OE of CP against *E.coli* (21.2 mm). On the contrary, minimum ZOI was observed for CE of CG and HF against *P. aeruginosa* (6.83 mm) and *K. pneumoniae* (6.23 mm) respectively. None of the extracts from HF showed activity against *E. coli, S. typhi* and *P. aeruginosa*. A detectable level of antibacterial activity was also not observed in CE of CP against *E. faecalis and S. pneumoniae*. The CE and OE of HF were also not effective against *S. pneumoniae*. No inhibition was observed in controls incubated with solvents and 10% acetic acid indicating that solvents and acetic acid themselves did not account for antibacterial activity observed in extracts of fish mucus. The OEs of CG, CP and HF showed maximum ZOI of 23.4 mm, 25.7 mm and 18.33 mm against *S. aureus, S. typhi* and *K. pneumoniae* respectively. Maximum ZOI of 17.3 and 10.13 mm was recorded against *S. aureus* in the CEs of CG and CP respectively. The CE of HF showed ZOI of 7.4 mm against *V. inaba.* Data analysis by one way ANOVA showed significantly different ZOI ($p<0.0001$) for various mucus extracts of CG, HF and CP on different bacterial strains tested.

Fish live in close interaction with pathogenic and non-pathogenic microorganisms in the aquatic environment and skin and its mucus secretion protect them from invading microorganisms by acting as a biochemical and physical barrier (Shephard, 1993). Previous studies (Hellio *et al.,* 2002; Kuppulakshmi *et al.,* 2008; Wei *et al.,* 2010; Kumari *et al.,* 2011) have explored the antibacterial properties of fish epidermal mucus, but there are scant studies on comparative antibacterial properties of different types of mucus extracts (CE, AE and OE) from air-breathing fishes. In the present study, mucus

Table 1. Antibacterial activity shown by mucus extracts of three fish species against different human bacterial pathogens

Bacterial strains	Diameter of zone of inhibition (mm)									
	Crude extract (CE) (10 µl)			Acidic extract (AE) (10 µl)			Organic extract (OE) (10 µl)			Standard antibiotic
	Fish species			Fish species			Fish species			
	CG	HF	CP	CG	HF	CP	CG	HF	CP	
Gram positive bacteria										Linezolid
E. faecalis	8.3±0.3^{a}	7.0±0.1^{b}	NA	15.0±0.2^{c}	24.3±0.3^{d}	8.8±0.2^{a}	9.0±0.2^{a}	7.1±0.2^{ab}	21.4±1.2^{e}	34.7±0.6^{f}
S. aureus	17.3±0.2^{a}	6.7±0.2^{b}	10.1±0.1^{c}	19.5±0.2^{d}	8.4±0.3^{e}	19.7±0.3^{d}	23.4±0.3	10.2±0.3^{c}	15.2±0.2^{g}	32±0.0^{h}
S. pneumoniae	8.0±0.1^{a}	NA	NA	10.8±0.2^{b}	7.9±0.2^{a}	7.0±0.2^{c}	8.5±0.3^{a}	NA	6.6±0.2^{c}	11.7±0.6^{d}
Gram negative bacteria										Imipenem
E. coli	12.1±0.2^{a}	NA	12.1±0.2^{a}	20.5±0.5^{b}	NA	21.2±0.3^{b}	21.0±0.9^{b}	NA	18.9±0.2^{b}	36.0±1.7^{c}
S. typhi	11.2±0.2^{a}	NA	8.0±0.0^{b}	22.9±0.2^{c}	NA	9.2±0.3^{d}	19.9±0.3^{e}	NA	25.7±0.2^{f}	38.7±1.2^{g}
K. pneumoniae	6.9±0.2^{a}	6.2±0.2^{a}	8.1±0.1^{b}	26.4±0.3^{c}	8.9±0.2^{b}	13.8±0.2^{d}	8.2±0.3^{b}	18.3±0.4^{e}	17.0±0.2^{f}	30.7±0.6^{g}
V. inaba	8.9±0.1^{a}	7.4±0.3^{b}	7.4±0.4^{b}	25.1±0.2^{c}	24.4±0.3^{c}	18.7±0.3^{d}	15.7±0.3^{e}	17.3±0.3^{f}	9.8±0.2^{a}	30.3±0.6^{g}
P. aeruginosa	6.8±0.15^{a}	NA	8.1±0.2^{b}	8.8±0.2^{b}	NA	8.5±0.4^{b}	7.4±0.3^{c}	NA	9.7±0.3^{d}	15.0±0.0^{e}

Values ar Mean±SD. Values with different subscripts in the same row are significantly different ($p<0.05$)
CG = *Clarias gariepinus*, HF = *Heteropneustes fossilis*, CP = *Channa punctatus*

was collected and extracted by three different methods in order to obtain different components which account for the differences in antibacterial activity of different mucus extracts. An acidic solvent extraction protocol was used to prepare a basic peptide /protein enriched mucus extract (Diamond *et al.,* 1991) which showed higher levels of antibacterial activity than OEs and CEs against *E. faecalis, S. pneumoniae, S. typhi, K. pneumoniae, V. inaba* and *P. aeruginosa* in CG, CP and against *E. faecalis, V. inaba* in HF. Subramanian *et al.* (2008) also compared AEs, OEs and aqueous extracts of haddock, brooktrout as well as hagfish and found AEs to be most effective against highly susceptible strain of *Salmonella enterica* C16. The AEs of *Channa striatus* (CS) showed a strong inhibitory effect against *P. aeruginosa, K. pneumoniae* and *B. subtilis* (Wei *et al.,* 2010). Skin mucus of CP and *Rita rita* extracted in 0.1% trifluoroacetic acid and 3% acetic acid showed strong inhibition against *S. aureus, M. luteus* and *S. typhi* (Kumari *et al.,* 2011).

In order to obtain mucus extract containing small molecules like secondary metabolites, the mucus was extracted using ethanol and solvent partitioned with DCM (Shapo *et al.,* 2007), which formed the organic extract (OE). The OEs of CG, CP and HF in the present study showed highest antibacterial activity against *S. aureus, S. typhi* and *K. pneumoniae* respectively. Subhashini *et al.* (2013) also reported that the OEs of tinfoil barb to be effective against *S. aureus, E. coli* and *Bacillus cereus*. Previous studies have also revealed the bactericidal activity of OEs against a wide range of pathogens (Liguori *et al.,* 1963; Hellio *et al.,* 2002). Subramanian *et al.* (2008) demonstrated that OEs of koi carp, brooktrout and striped bass have bacteriostatic activity.

CE was tested for its antibacterial activity as it contains all the components such as immunoglobulins, calmodulins, lectins, glycoproteins, lysozymes, flavoenzymes, carbonic anhydrases, peptides and proteolytic enzymes (Shepherd, 1993 Kitani *et al.,* 2008). CEs exhibited less antibacterial activity than AEs and OEs in the present study which may be because of the presence of the components such as carbonic anhydrases (Supuran and Capasso, 2017) and calmodulins having no antibacterial activity. No activity was shown by CEs of CP against *S. pneumoniae* in the present study. CEs of HF also showed no antibacterial activity against *S. pneumoniae, E. coli, P. aeruginosa* and *S. typhi.* CEs of CP exhibited ZOI of 8.06 mm, in our study against *K. pneumoniae* showing antibacterial activity which is contrary to the report of Dhotre *et al.* (1963), who reported no activity against the same bacteria. Our findings are in close agreement with Kuppulakshmi *et al.* (2008) who also found that CEs of CP was effective against *S. aureus, E. coli, S. typhi, K. pneumoniae* and *P. aeruginosa.* Crude mucus of *Hypophthalmichthys nobilis* also showed strong inhibition against *E. coli, B. Cereus, K. pneumoniae, S. aureus, S. epidermidis, P. aeruginosa* and *S. aureus* (Tyor and Kumari, 2016). On the contrary, CEs of CS showed no activity against *Bacillus subtilis, K. pneumoniae*, *Salmonella enteritidis*, *Proteus vulgaris* and *P. aeruginosa* (Wei *et al.,* 2010). Strong antibacterial activity of eel CEs was reported by Bragadeeswaran and Thangraj (2011) against *S. aureus, E. coli, P. aeruginosa* and no activity against *K. pneumoniae*.

Most of the mucus extracts of CG, HF and CP in the present study showed antibacterial activities suggesting the existence of one or more antibacterial constituents. Significant variation was observed in the antibacterial activity of mucus extracts from the three different fish species. These variations may be due to different components separated by different solvents. Maximum antibacterial activity in the present study was recorded in AEs which may be due to the presence of basic peptides in the extract. The antibacterial activity observed in the OEs may be attributed to the the polarity of the extracts (Hellio *et al.,* 2002). Fish mucus glycoproteins may also be responsible for its antibacterial activity because they have the ability to form holes in the bacterial membrane (Rao *et al.,* 2015). Antibacterial activity of the different extracts of the three freshwater fish species varied remarkably against the 8 bacteria in the present study, which may be due to the difference in the viscosity of mucus produced by the epithelial and epidermal layer of the three fish species, which could alter the composition of mucus. Physiological and ecological conditions of the donor fish such as, stages of growth and maturity, handling stress, PH and salinity may also contribute towards the difference in the antibacterial activity (Blackstock and Pickering, 1982; Lebedeva, 1999). Antibacterial activity of the different mucus extracts *viz.* CEs, AEs and OEs of the three freshwater fish species (CG, HF and CP) varied significantly ($p<0.05$) against the 8 human pathogenic bacteria. Maximum antibacterial activity was observed by AEs of *C. gariepinus* against Gram negative bacteria. The order of the level of antibacterial activity of the mucus from the three fish species observed in the present study was: *C. gariepinus* > *C. punctatus* > *H. fossilis* and the acidic extract of *C. gariepinus* showed the maximum antibacterial activity against Gram negative bacteria. Therefore, epidermal mucus from fish skin could be a source of novel antimicrobial components against human bacterial pathogens. Further studies are needed to analyse the antibacterial activity in comparison with chemical composition of mucus.

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