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Defense properties in the epidermal mucus of different freshwater fish species

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Abstract. Mucus is a protective secretion of the epidermal membranes that cover the surface of fish as the first line of defense against invading pathogens. The exploration of the defence properties of fish mucus is limited and scarcely reported in a few marine and freshwater fish species. To date, no studies have shown the defence properties of fish mucus in Malaysian freshwater fish species such as giant snakehead fish (*Channa micropeltes*), striped snakehead (*Channa striatus*), tilapia (*Oreochromis niloticus*) and bagrid catfish (*Mystus nemurus*). In this study, a series of extraction solvents (acidic, organic and aqueous) were utilized to screen for antimicrobial activity of the epidermal mucus for the above stated freshwater fish species. Preliminary screening of the mucus extracts against *Escherichia coli* showed significant variation in antimicrobial activity among the fish species examined. Acidic mucus extracts of tilapia and bagrid catfish exhibited bactericidal activity. No detectable antibacterial activity was noted in the crude and organic mucus extracts of all the fish species. Based on the preliminary screening analysis, minimum bactericidal concentration (MBC) for acidic mucus extract of tilapia and bagrid catfish against a fish pathogen and nine human pathogens were determined. Acidic mucus extracts of bagrid catfish have showed the lowest MBC values (11.96 µg mL^{-1}) against the Gram-negative bacteria. The data suggests that the mucus of tilapia and bagrid catfish may be a source of novel antimicrobial agents for fish and human health related applications.
Key Words: mucus, antimicrobial activity, *Channa striatus*, *Oreochromis niloticus*, *Channa micropeltes*, *Mystus nemurus*.

Introduction. In fish, the epidermal mucus is the external barrier between the environment and fish which is considered as a key component of innate immunity (Ingram 1980). The epidermal mucus is produced primarily by epidermal goblet or mucus cells and is composed mainly of water and gel-forming macromolecules, including mucins and other glycoproteins (Shephard 1993). These cells start to differentiate in the basal part of the epidermis, and then grow in size and move towards the surface where they release their content (Pickering 1977). The mucus layer covers the surface of external body to reduce body friction against water and to protect from abrasion injury. The epidermal fish mucus makes the surfaces smooth and slippery and has mechanical protective nature (Cameron & Endean 1973). However, the composition and rate of mucus secretion has been observed to change in response to microbial exposure or to environmental perturbations such as hyperosmolarity and acidity (Ellis 2001).

Fish epidermal mucus contains water, lipids, amino acids, secretory proteins, glycoprotein, sloughed skin cells and bacteria (Shephard 1994). The mucus layer on the fish surface performs a number of formidable functions, including disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding and nest building (Shephard 1994). An intricate array of both specific and innate immune components have been identified and characterized in fish. Key innate immune

components include the mucus layer on the skin, gills and gastrointestinal tract, and also constituents of blood such as phagocytes and natural killer cells (Arockiaraj et al 2013a). The mucus layer is continuously replaced, which prevents the stable colonization of potential infectious microorganisms and invasion of metazoan parasites (Nagashima et al 2003; Arasu et al 2013). As a component of the innate immune mechanism, the mucus plays a dual role. First, by being continuously produced and sloughed off, it prevents pathogen adherence (Arockiaraj et al 2013b). It also contains a variety of biologically active substances that function as innate immune factors (Subramanian et al 2007, 2008; Palaksha et al 2008; Arockiaraj et al 2014).

Fish mucus also serves as a repository of numerous innate immune factors such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, proteolytic enzymes and various other antibacterial proteins and peptides (Shephard 1994; Cole et al 1997; Arasu et al 2014). Immunoglobin M (IgM) type natural antibody has also been found in mucus secretions of the skin and gut, as known to have involved in neutralization of pathogens, complement activation, opsonization and in hypersensitivity responses (Roberts 2001). IgM antibodies were found in the skin mucus of olive flounder (Palaksha et al 2008). Trypsin with strong bactericidal activity against Gram-positive bacteria was reported from the skin mucus of rainbow trout (*Oncorhynchus mykiss*) (Hjelmeland et al 1983) and Atlantic salmon (*Salvelinus alpinus*) (Ross et al 2000). All these natural antibodies, antibacterial agglutinin, trypsin-like protease transferrin and lysozyme are the major mucosal immune components of fish (Palaksha et al 2008). Therefore, fish mucus is considered as one of the important components of first line of defence against infectious pathogens.

The antimicrobial property of crude epidermal mucus against infectious pathogens was initially demonstrated in rainbow trout (Austin & McIntosh 1988). The removal of epidermal mucus from ayu (*Plecoglossus altivelis*) and turbot (*Scophthalmus maximus*) after challenging them with *Listonella anguillarum* resulted in increased mortality (Fouz et al 1990). In common carp (*Cyprinus carpio*), the loss of epidermal mucus, increased the susceptibility to bacterial infection (Lemaitre et al 1996). Antimicrobial activity of epidermal mucus extracts against a broad range of microbial pathogens was observed by Hellio et al (2002). These experiments supported the hypothesis that the epidermal mucus has protective function against microbial infection in fish. The exploration of the antimicrobial properties of fish mucus is limited and scarcely reported in a few marine and freshwater fish species. To date, no studies have shown the antimicrobial properties of fish mucus in Malaysian freshwater fish species such as giant snakehead fish (*Channa micropeltes*), striped snakehead (*Channa striatus*), tilapia (*Oreochromis niloticus*), and bagrid catfish (*Mystus nemurus*). In this study, a series of extraction solvents (acidic, organic and aqueous) were utilized to screen for antimicrobial activity of the epidermal mucus for the above stated freshwater fish species.

Material and Method

Fish and their maintenance. Mucus samples were obtained from four freshwater fish species (giant snakehead fish, striped snakehead, tilapia and bagrid catfish). All the fishes were obtained during January 2014 from local fish farm, Sungai Petani, Kedah Darul Aman, Malaysia. Fish were maintained at the aquaculture laboratory, Faculty of Applied Sciences, AIMST University. All the fish species were kept separately in 500 L circular cement tanks. Striped snakehead, tilapia and bagrid catfish were stocked at 10 fish per tank, while giant snakehead fish were stocked at 6 fish per tank. The water temperature (mean ± SD) was 28.8±0.56^{o}C and pH was 6.7±0.13. Tilapia and bagrid catfish were fed *ad libitum* with commercial feed twice daily whereas giant snakehead and snakehead were fed with small live trash fishes (*Rasbora* spp) daily. The mean fish body weights were 640±168 g, 320±154 g, 116±50 g, and 515±58 g for giant snakehead, snakehead, tilapia, and bagrid catfish respectively. The fish tanks were cleaned and water was changed daily to maintain good water quality and to avoid microbial infection. Dead fish or fish with lesions were removed immediately from the tanks. Only healthy fish were chosen for sampling.

Mucus collection and extraction. Mucus collection was done with certain modifications following the method of Subramanian et al (2008). Only healthy fish were chosen for mucus collection and fish with lesions were discarded. Prior to mucus collection the fish were starved for 24 hours, and they were anaesthetized with a sub-lethal dose of 100 mg L^{-1} of MS-222 (Tricaine methanesulphate, Sigma, USA). The fish were transferred individually into a sterile polyethylene bag followed by addition of 5 to 10 mL of 50 mM NaCl into the bag immediately. The fish was gently moved back and forth inside the bag for 3 to 5 min to slough off the mucus and then the fish was released into the tanks. Mucus was obtained from 10 individuals from each species and mucus samples were pooled. The pooled mucus sample was then divided into three parts, which were extracted separately with acidic and organic solvents.

The acidic extracts of mucus were prepared using a modified method of Diamond et al (1991). Immediately after collection, 150 mL of the pooled mucus sample was mixed with 150 mL of 10% (v/v) glacial acetic acid and placed in a boiling water bath for 5 min. The acid-mucus mixture was placed in ice, homogenized and centrifuged at 18,000 ×g (Beckman coulter, Avanti J-26 XPI) for 35 min at 4°C. The supernatant was collected and partially purified using a reverse-phase Sep-Pak Vac 5g C18 cartridge. Prior to the addition of supernatant, the cartridge was activated with 30 mL of methanol and equilibrated with 10 mL of 10% (v/v) acetic acid. After loading the supernatant, the cartridge was washed with 10 mL of 0.1% (v/v) trifluoroacetic acid (TFA) and then eluted with 40 mL of an acetonitrile/water/TFA (80.0:19.9:0.1, v/v/v) mixture. The resulting elutes were then condensed in DNA Concentrator (Centrivap DNA Concentrator) and re-suspended in water and then assayed for antimicrobial activity.

The organic extracts of mucus were prepared as described by Hellio et al (2002) with slight modifications. The pooled mucus sample (150 mL) was frozen immediately and freeze-dried (Freeze Dryer Supermodulyo-based High Capacity System). The dried mucus powder was suspended in 95% ethanol (HmbG Chemicals, Germany) at 10 mg mL^{-1} and centrifuged at 11,000 ×g (Beckman coulter, Avanti J-26 XPI) for 30 min at 4°C. The supernatant was decanted and the pellet was re extracted for twice. The ethanol extracts were combined and evaporated in DNA Concentrator (Centrivap DNA Concentrator). The extract was re-suspended in 50 mL of distilled water and partitioned four times with 200 mL (4×50 mL) of dichloromethane (DCM). The aqueous phase was freeze dried (Freeze Dryer Supermodulyo-based High Capacity System), while the DCM phase (organic) was pooled and evaporated in DNA Concentrator (Centrivap DNA Concentrator). The dried mucus samples obtained from the aqueous and organic phases were re-dissolved in water and 5% (v/v) dimethyl sulphoxide (DMSO) respectively and assayed for antimicrobial activity.

The crude extracts of mucus were prepared using a modified method of Subramanian et al (2008). To prepare crude extracts, the pooled mucus (150 mL) was freeze-dried, re-suspended in water at 10 mg mL^{-1} and centrifuged at 9500 ×g (Beckman coulter, Avanti J-26 XPI) for 10 min at 4 °C. The supernatant was collected and stored in -20^{o}C until further antimicrobial assay.

Bacterial pathogens and their culture conditions. Mucus extracts were tested against a range of human pathogens and a fish pathogen for antimicrobial activities including both Gram-positive bacteria (Methicillin-Resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), *Micrococcus luteus* (ATCC 4698), *Bacillus subtilis* (ATCC 11774), *Bacillus cereus* (HQ 185283) and Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Salmonella enterica serovar typhimurium* (IMR-S391), *Salmonella enterica serovar enteritidis* (IMR-S966), *Klebsiella pneumonie* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), and a fish pathogen, *Aeromonas hydrophila* (ATCC 49140). The bacteria were maintained in the glycerol stock in Difco Luria Bertani (LB) broth except *M. luteus* and *A. hydrophila* were maintained in Nutrient Broth (NB) with 80% glycerol stock and was kept in -80^{o}C. For experimental purposes, the bacterial strains were revived from glycerol stocks by inoculating a loopful of culture and streaking onto LB agar plates and incubated in Thermo Scientific Type BK 6160 incubator at 37^{o}C. The overnight cultures were then sub cultured again onto LB agar and incubated overnight at 37^{o}C. A single

colony was picked from the plate with a sterile wire loop and inoculated in Muller Hinton broth for antimicrobial testing for each bacterium.

Screening of mucus extracts for antimicrobial activity. Preliminary screening for antimicrobial activity of the acidic, organic and aqueous mucus extracts of giant snakehead, striped snakehead, tilapia and bagrid catfish was carried out against *E. coli* (ATCC 25922) that has shown a high susceptibility to various antibiotics and antimicrobial peptides. The antimicrobial activity was studied using the broth micro-dilutions following the slightly modified method of Subramanian et al (2008). Assays were carried out in triplicate for each mucus extracts in a 96-well plate (Greiner Bione, Cellstar) for both volumes. The bacteria (*E. coli*) was grown overnight (18-24 hours) at 37^{o}C to mid-logarithmic and diluted to a final density of 2 X 10^{4} mL. Thirty µL of MH broth were added into each well and 20 µL of diluted bacterial culture was added to the wells containing mucus extracts respectively. The controls also were assayed with mucus extracts in the microtiter plate. For a positive control, 100 µL of mucus extracts, a 130 µL of MH broth were incubated with 20 µL of inoculum and for negative control, a 100 µL of solvents which is used to dissolve the mucus extracts were two-fold diluted with MH broth then 30 µL of MH broth added and incubated overnight (18-24 hours) at 37^{o}C with 20 µL of inoculum. The antimicrobial activity was confirmed by visual inspection, absorbance at 595 nm using microplate reader (Tecan Infinite M 200 Pro). The antimicrobial activity was further confirmed by spread plating on MH agar plates. The minimal bactericidal concentrations (MBCs) of mucus extracts were defined as the minimum mucus concentration (µg mL^{-1}) that caused a complete inhibition of bacterial growth. The potential mucus extracts exhibiting antimicrobial activity were further tested against other fish and human pathogens using the broth dilution method as mentioned above (Table 1).

Table 1
List of bacterial pathogens and their reference number used for antimicrobial activity test

Strain name	*Reference number*
Gram-negative	
Escherichia coli	ATCC 25922
Salmonella enterica serovar typhimirium	IMR S391
Salmonella enterica serovar enteritidis	IMR S966
Klebsiella pneumonia	ATCC 700603
Aeromonas hydrophila	ATCC 49140
Pseudomonas aeruginosa	ATCC 27853
Gram-positive	
Methicillin-resistant Staphylococcus aureus	ATCC 33591
Micrococcus luteus	ATCC 4698
Bacillus subtilis	ATCC 11774
Bacillus cereus	HQ 185283

Protein quantification and SDS Page. The protein quantification for mucus extract was carried out based on Bradford protein assay (Bradford 1976) by using bovine serum albumin (BSA) (Sigma, USA) as a protein standard. The protein profile of acidic mucus was examined using tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE) as described by Oren & Shai (1996). Protein samples (15 µg total protein) were diluted 1:1 with sample buffer [4% (w/v) SDS, 50 mM Tris–HCl, 2% mercaptoethanol (v/v), 12% (v/v) glycerol and 0.5% (w/v) bromophenol blue adjusted with HCl to pH 6.8] and loaded onto a 16% acrylamide with a 10% spacer and 4% stacking gel. SDS-PAGE standard markers (unstained low range-Thermo scientific) were included to estimate the molecular mass of proteins. The gel was run in a BioRad electrophoresis apparatus for 1.5–5.5 hours at 30 V during initial voltage then followed by 150 V until 2 mm from the base of the gel. Then the gel electrophoresis was stopped and fixed with fixation solution for 30 minutes then followed by staining with Coomassie

Brilliant Blue for at least 12 hours with gentle agitation. Then the gel was destained twice in 10% acetic acid for another 15-60 minutes followed by visualization using Gel-Doc XR System, (Biorad Laboratories, USA).

Statistical analysis. One way analysis of variance and Duncan's multiple-range tests were employed to analyze data collected for protein content in different fish mucus. Differences between means were considered significant when $p < 0.05$.

Results

Protein content in different mucus extracts. The protein content of the mucus extracts of four different freshwater fish species (giant snakehead, tilapia, striped snakehead and bagrid catfish) were presented in Table 2. In the crude extracts, significantly highest protein content (579.9±32.3 µg mL^{-1}) was observed in tilapia fish followed by giant snakehead (535.2±93.1 µg mL^{-1}) and bagrid catfish (466.3±53 µg mL^{-1}) while the lowest protein content (432.9±28.2 µg mL^{-1}) was observed in striped snakehead. For the acidic extracts, the highest protein content (239.3±7.8 µg mL^{-1}) was also observed in tilapia followed by bagrid catfish (179.3±16.2 µg mL^{-1}) and giant snakehead (86.4±32.2 µg mL^{-1}) and the lowest protein content (53.4±2.0 µg mL^{-1}) was observed in striped snakehead. For the aqueous phase extracts, tilapia fish mucus showed the highest protein content (56.90±4.1 µg mL^{-1}) and the lowest protein content was observed in bagrid catfish (4.9±0.5 µg mL^{-1}). The protein contents in giant snakehead and striped snakehead were found to be 27.2±8.8 and 6.2±1.3 µg mL^{-1}, respectively. For dichloromethane (DCM) phase mucus extracts, tilapia contained higher protein content (31.8±6.3 µg mL^{-1}) than the other fish species (Table 3). The lowest protein content was noticed in bagrid catfish (4.4±1.1 µg mL^{-1}), followed by giant snakehead (6.7±4.1 µg mL^{-1}), and the highest was found in striped snakehead (26±7.9 µg mL^{-1}).

Table 2

The protein content (mean±SD) of different mucus extracts of four freshwater fish species [giant snakehead, tilapia, striped snakehead and bagrid catfish, n= 10 except giant snakehead where n=6]

Fish species	*Crude (µg mL^{-1})*	*Acidic (µg mL^{-1})*	*Aqueous (µg mL^{-1})*	*DCM (µg mL^{-1})*
Giant snakehead	535.20±93.10	86.40±32.20	27.20±8.80	6.70±4.1
Tilapia	579.90±32.30	239.30±7.80	56.90±4.10	31.80±6.30
Striped snakehead	432.90±28.20	53.40±2.00	6.20±1.30	26.00±7.90
Bagrid catfish	466.30±53.00	179.30±16.20	4.90±0.50	4.40±1.10

Each value is the mean and standard deviation of three replicates. Values followed by a different superscript letter on the same column are significantly different ($p < 0.05$)

Table 3

Preliminary screening of crude, acidic, aqueous and dichloromethane (DCM) phase extracts against *E. coli* (ATCC 25922) for 100 µL of mucus sample

	Crude extract			*Acidic extract*			*Aqueous phase extract*			*DCM phase extract*		
	R1	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*
Giant snakehead	-	-	-	-	-	-	-	-	-	-	-	-
Tilapia	-	-	-	+	+	+	-	-	-	-	-	-
Striped snakehead	-	-	-	-	-	-	-	-	-	-	-	-
Bagrid catfish	-	-	-	+	+	+	-	-	-	-	-	-
+ ve	-	-	-	-	-	-	-	-	-	-	-	-
- ve	-	-	-	-	-	-	-	-	-	-	-	-

+ indicates antimicrobial activity; - indicates no antimicrobial activity.

Preliminary screening for antimicrobial activity of mucus extracts. The results obtained from the test with 100 µL of mucus extract revealed that the acidic extracts of tilapia and bagrid catfish had potent bactericidal activity, while the giant snakehead and striped snakehead showed no detectable antimicrobial activity against *E. coli* (Table 2). There was no antimicrobial activity observed among the rest of the extracts against a control strain *E. coli.*

Minimum bactericidal concentration (MBC). The acidic extracts of tilapia and bagrid catfish were further assayed to determine minimum bactericidal concentration (MBC) against a wide range of Gram-positive and Gram-negative bacteria. Acidic mucus extracts of tilapia and bagrid catfish have showed a broad spectrum of bactericidal activity. The bactericidal concentration of acidic mucus extracts was determined by streaking aliquots of the assay contents on Mueller Hinton (MH) agar plates. In the acidic extracts, the minimum concentration of mucus protein that resulted in no viable growth was taken as the minimum bactericidal concentrations (MBC) (Table 4). The controls were incubated with solvents and bacterial culture showed negative results, demonstrating that the solvents themselves did not account for the antimicrobial activity that observed in acidic fish mucus extracts. The minimum bactericidal concentrations of mucus from tilapia and bagrid catfish were found to vary for each pathogenic microbe. Each of these fish mucus also showed varied activities towards different bacteria.

Table 4

Minimum bactericidal concentration (MBC) of active acidic mucus extracts of tilapia and bagrid catfish

Name of pathogens and reference number	*Minimum bactericidal concentration (MBC) (µg protein mL^{-1})*	
	Tilapia	*Bagrid catfish*
Gram-negative		
Escherichia coli (ATCC 25922)	15.96	23.91
Salmonella enterica serovar typhimirium (IMR S391)	31.91	-
Salmonella enterica serovar enteritidis (IMR S966)	31.91	23.91
Klebsiella pneumoniae (ATCC 700603)	31.91	23.91
Aeromonas hydrophila (ATCC 49140)	31.91	23.91
Pseudomonas aeruginosa (ATCC 27853)	-	23.91
Gram-positive		
Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591)	31.91	11.96
Micrococcus luteus (ATCC 4698)	15.96	11.96
Bacillus subtilis (ATCC 11774)	15.96	11.96
Bacillus cereus (HQ 1852830)	31.91	11.96

(-) indicates no inhibition.

The minimum bactericidal concentration (MBC) in the mucus extracts of all the fish species against different pathogens ranged between 11.96 and 31.91 µg mL^{-1}. The acidic mucus extracts of tilapia inhibited at the lowest bactericidal concentration and MBC values (15.96 µg mL^{-1}) against *E. coli, M. luteus* and *B. subtilis.* The higher MBC values was observed in other pathogens such as *S. enterica serovar typhimirium, S. enterica serovar enteritidis, K. pneumoniae, A. hydrophila*, Methicillin-resistant *S. aureus* (MRSA) and *B. cereus*.

The MBC from acidic extracts of bagrid catfish mucus against Gram-positive microbes such as Methicillin-resistant *S. aureus*, *M. luteus*, *B. subtilis*, *B. cereus* was found to be 11.96 µg mL^{-1}. The two times higher MBC values was observed in Gram-negative pathogens (*E. coli, S. enterica serovar enteritidis, K. pneumoniae, A. hydrophila* and *P. aeruginosa*) than Gram-positive pathogens. There were no bactericidal activities observed in acidic mucus extracts of bagrid catfish against *S. enterica serovar typhimirium* and acidic mucus extracts of tilapia against *P. aeruginosa*. In summary,

acidic extract of tilapia and bagrid catfish mucus exhibited the lowest MBC values of 15.96 µg mL^{-1} and 11.96 µg mL^{-1}, respectively and inhibited all the tested pathogens.

The protein profiles of the active acidic extracts of tilapia and bagrid catfish were showed in Figure 1. The Tricine SDS-PAGE profile showed the protein ranging from 100 kDa to less than 10 kDa. The tilapia and bagrid catfish acidic extracts showed a few high molecular mass protein bands, but the low molecular mass proteins below 15 kDa were more prominent. The more intense protein bands were noticed in all the fish species with the molecular mass protein less than 15 kDa. The acidic extracts of tilapia showed two protein bands at 14.34 kDa to 10.24 kDa while bagrid catfish also showed one protein band at 13.04 kDa, respectively.

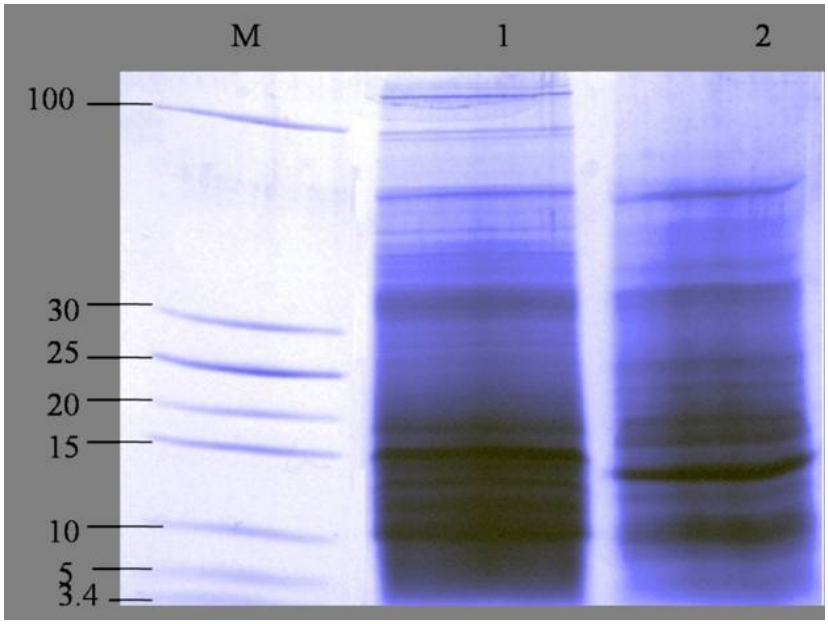

Figure 1. Tricine SDS-PAGE showing protein profile of acidic mucus extract from two fish species. 1. Tilapia and 2. Bagrid catfish. M - Low range molecular mass (kDa) marker. Each lane contains of 15 µg acidic mucus extracts.

Discussion. In the aquatic environment, fish are constantly exposed to wide range of pathogenic and non-pathogenic microorganisms (Shephard 1994). Fish epidermal mucus plays a vital role in maintaining fish health by providing physical and biochemical barriers between the fish and the environment. Fish mucus has been proven to play a major role in the prevention of colonization by bacteria, fungi and parasites (Fouz et al 1990). Several studies have been demonstrated the protective role of mucus and its components in various fish species (Nagashima et al 2001; Sarmasik 2002). These studies suggested that the epidermal mucus acts as a first line of defense against pathogens. This epidermal mucus layer on the fish also performs other functions such as disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding and nest building (Shephard 1994). There have been numerous studies on innate immune factors in fish epidermal mucus, including the role of proteases and antibacterial agents (Fast et al 2002; Subramanian et al 2007; Hellio et al 2002), suggesting that the epidermal fish mucus can inhibit the growth of bacteria and therefore, may have a potential source of novel antimicrobial components in it. Several studies have been carried out to explore the properties and antimicrobial components of different fish mucus, to date little information is available for the antimicrobial properties of epidermal mucus of the fishes such as *C. micropeltes*, *C. striatus*, *O. niloticus* and *M. nemurus* and hence the present study was carried out to assess the antimicrobial activities of the epidermal mucus of the above important fish species for better health management.

In the present study, epidermal mucus was collected and extracted with acidic, organic and aqueous solvents to obtain different components of the mucus. Protein quantification results revealed that crude mucus extracts of all fish species contained a

high amount of proteins compared to other extracts. Among the fish mucus, tilapia contained more protein in all the extraction methods compared to those in other fish species examined in the study. The biochemical substances of mucus have been shown to differ depending on the ecological and physiological conditions such as salinity, pH, handling stress and stages of growth and maturity (Loganathan et al 2011). The variation in the amount of mucus secretion between fish species have been observed to change during infection.

Antimicrobial screening results showed that no detectable levels of antimicrobial activity observed in crude and aqueous mucus extract against human pathogens tested. Among the 16 mucus extracts that were prepared, only acidic mucus extracts of tilapia and bagrid catfish showed highest levels of antimicrobial activity in the preliminary screening against *E. coli* (ATCC 25922) as a control strain (Fass & Barnishan 1979).

The dichloromethane (DCM) extracts from the mucus of brook trout (*Salvelinus fontinalis*), koi carp (*Cyprinus carpio*) and striped bass (*Morone saxatilis*) demonstrated bacteriostatic activity to control the growth of bacteria without killing them. Hellio et al (2002) reported that organic extracts of fish mucus show bactericidal activity against a broad range of pathogens and such promising activity was not observed in the present study. These results indicated that the small molecules extract via organic solvents, may not be the most active antimicrobial components in the mucus of the examined fish species. None of the crude and aqueous extracts showed detectable levels of antimicrobial activity against *E. coli*. Earlier studies demonstrated, no microbial growth inhibition in aqueous fish mucus extracts of a wide range of fish species including Arctic char, brook trout, koi carp, striped bass, haddock (*Melanogrammus aeglefinus*) and hagfish (*Myxine glutinosa*) (Subramanian et al 2008). Further, the antimicrobial activity of epidermal mucus extracted with acidic, organic and aqueous solvents varies remarkably within and among the fish species (Subramanian et al 2008). The variation in antimicrobial activities among different fish species in this study is thought to be due to the diverse composition of the secreted mucus. The mucus producing cells in epidermal and epithelial layer of fish had been reported to differ between fish species (Shephard 1993) and therefore, could influence the mucus composition. Bragadeeswaran & Thangaraj (2011) reported that the crude mucus extracts of eel fish (*Anguilla anguilla*) could exhibit antimicrobial activity. The absence of antimicrobial activity of the aqueous extracts in this study could be due to the presence of low levels of enzymes in the mucus extract (Subramanian et al 2008). It has been reported that mucus enzymes may influence the innate defence by activating the expression of genes that encode proteins, such as antimicrobial peptides and complement proteins and could thereby impart antimicrobial activity through indirect mechanism. For example, cathepsin D and matrix metalloprotease have been shown to be involved in the production of the antimicrobial peptide (parasin I) in the mucus of catfish (*Parasilurus asotus*) (Cho et al 2002).

Further, the acidic mucus extract of tilapia and bagrid catfish were found to inhibit most of the human pathogens such as *E. coli, S. enterica serovar typhimirium, S. enterica serovar enteritidis, K. pneumoniae, P. aeruginosa,* Methicillin-resistant *S. aureus* (MRSA), *M. luteus, B. subtilis* and *A. hydrophila*. Similarly, the acidic mucus extracts of brook trout, haddock and hagfish showed bactericidal activity against a wide range of fish and human pathogens (Subramanian et al 2008). This suggests that antimicrobial components in the acidic mucus extracts may have a key role in host defence against pathogenic infection in the aqueous environment. Previous studies have shown a variety of antimicrobial proteins such as (paradaxin and pleurocidin) from fish mucus that is potentially involved in the protective function against invading pathogens (Cole et al 1997).

The minimum bactericidal concentration (MBC) was observed in the range of 11.96 to 23.91 for bagrid catfish and 15.96 to 31.91 µg protein mL^{-1} for tilapia against a wide range of pathogens. This study demonstrated a low MBC value against Gram-positive bacteria than Gram-negative. The acidic extracts were highly active against both Gram-positive (Methicillin-resistant *S. aureus* (MRSA), *M. luteus, B. cereus* and *B. subtilis*) and Gram-negative (*E. coli*, *S. enterica serovar typhimirium*, *S. enterica serovar enteritidis*, *K. pneumoniae*, *A. hydrophila* and *P. aeruginosa*). Previous studies have

reported MBCs which were within the range of 15 to 115 µg protein mL^{-1} in the mucus extracts of eel, tench (*Tinca tinca*), rainbow trout, turbot and carp (Ebran et al 1999), and 180 µg protein mL^{-1} in rockfish (*Sebastes schlegeli*) extracts (Nagashima et al 2003).

The acidic mucus extract of hagfish showed antimicrobial activity in the range of 6 to 82 µg protein mL^{-1}. However when compared with the range acidic extracts of bagrid catfish and tilapia showed much lower. Acidic extracts, of tricine SDS-PAGE, showed more prominent bands in low molecular mass. Hence, this could be a potent antimicrobial peptides fall in the range of low molecular mass proteins of the tricine SDS-PAGE and could be a novel source of antimicrobial peptide.

Conclusions. The present study reveals that the acidic extracts of epidermal mucus of *O. niloticus* and *M. nemurus* exhibit antimicrobial activity against *E. coli* in the preliminary screening. The acidic mucus extracts of these fish species were then used for the determination of minimum bactericidal concentration (MBC) where the mucus extract of tilapia and bagrid catfish showed MBC value in the range of 11.96 to 31.91 kDa. This indicated that this acidic mucus extract highly susceptible for Gram-positive and Gram-negative pathogens. In tricine SDS-Page analysis showed that the antimicrobial prominent band was found to be in low molecular masses.

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