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Biotic potential of mucus extracts of giant mudskipper *Periophthalmodon schlosseri* (Pallas, 1770) from Pichavaram, southeast coast of India

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Abstract

Background: The epidermal mucus of fish contains various bioactive compounds, which play a crucial role in the animal defense mechanisms. However, the mucus from giant mudskipper is not well characterized for its biochemical and pharmacological effects. This study for the first time analyzed the mucus extracts of this species for antimicrobial (against human pathogen) and hemolytic activities. Fourier-transform infrared spectrum analysis (FT-IR) and SDS-PAGE of mucus extracts were also carried out.

Results: The protein content in *Periophthalmodon schlosseri* (Gobiidae: Oxudercine) mucus extracts was 0.66 ± 0.04 mg/ml. The mucus extracts of *P. schlosseri* showed antibacterial activity against all eight human pathogenic bacteria and four fungal strains tested in this study. The hemolytic activities against chicken and human blood cells were observed with maximum activity for dichloromethane extracts. The FT-IR spectrum of mucus extract of *P. schlosseri* showed the presence of functional groups, evident protein bands of various molecular weights ranging from 14 kDa to 97 kDa.

Conclusion: The obtained results suggested that the mucus of this species has proteinaceous compounds responsible for antimicrobial and hemolytic activities.

Keywords: *Periophthalmodon schlosseri*, Antimicrobial, Hemolytic, SDS-PAGE, FT-IR

Background

Fish and fish products are rich in high valuable proteins, minerals, enzymes, and pigments (Hellio, Pons, Beaupoil, Bourgougnon, & Le Gal, 2002). Fish lives in an environment which is a source of microorganisms that can be potentially pathogenic. Fish can still preserve a fit and vigorous system under normal condition. Skin or mucus of fish contains biologically active compounds with a potential against human pathogens. The mucus is composed of chemical substances secreted from the epithelial cells covering the epidermal surface (Rakers et al., 2013). Mucus contains mucin-like substances which have an antibiotic potential (Ingram, 1980) that reduces the adhesive potential of bacteria present in mucus and is believed to be a key virulence factor (Ellis, 2001).

The mucus substances produced by fish act as a lubricant (Ellis, 1999; Shephard, 1994). The mucus layer acts as a barrier between fish and its aquatic environment and is presumed to be involved in a number of certain functions including immunological, respiratory, osmosis, locomotory, reproductive, signaling, foraging, and nesting (Uribe, Folch, Enriquez, & Moran, 2011). Proteinaceous slimy discharge of the fishes contains antibiotic peptides, as their first developed basics of innate immunity defense line and they act as a natural resistance of animals against microorganisms (Balasubramanian, Baby Rani, Prakash, & Prakash, 2012; Manivasagan, Annamalai, Ashokkumar, & Sampathkumar, 2009). The aqueous extracted mucus of fishes demonstrated the presence of various innate antimicrobial agents such as lysozyme, cathepsin B, and

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trypsin-like proteases (Caruso et al., 2010; Caruso et al., 2014; Subramanian, Mackinnon, & Ross, 2007), and it may be a source of novel antimicrobial agents for fish and human health-related applications.

The antimicrobial property of epidermal mucus in fishes is reported by various researchers from throughout the world: common carp (*Cyprinus carpio*) by Lemaître et al. (1996); hagfish (*Myxine glutinosa*) by Subramanian, Rose, and Mackinnon (2008); catfish (*Arius maculatus*) by Manivasagan et al. (2009); blue-spotted mudskipper (*Boleophthalmus boddarti*) by Ravi, Kesavan, Sandhya, and Rajagopal (2010); and eel fish (*Anguilla anguilla*) Caruso et al. (2010 and 2014).

Mudskippers (Gobiidae: Oxudercine) inhabits the intertidal mudflats and mangrove areas where they live under varying environmental conditions (tide, wind, temperature, pH, and hypoxic soils) and they have certain various physiological adaptations for their survival (Graham & Lee, 2004). Mudskippers can secrete slime to protect the skin from scratch and dehydration. Discovery of novel drugs is the most significant biomedical current research and is due to untreatable diseases as well as increased resistance to current therapeutic treatments of many bacterial pathogens. In biosynthetic biomedical exploration, the marine natural products (MNPs) such as new antibiotics are still a high priority in biosynthetic biomedical exploration, and therefore, in the present study, the mucus extracts of *P. schlosseri* were screened to evaluate their antimicrobial and hemolytic properties.

Methods

Sample collection and preparation of mucus extract

Specimens of the giant mudskipper *Periophthalmodon schlosseri* were freshly collected from Pichavaram, southeast coast of India (lat. 11° 26′ N, long.79° 48′ E). Fishes were anesthetized and euthanized with a lethal dose of MS-222 (tricaine methanesulfonate, 0.1 g l^{-1}, Sigma-Aldrich). Samples of skin mucus were collected from fish by gently scraping with a sterile spatula from the dorsal surface of the body; the ventral side of the body was not considered for the same in order to avoid intestinal and sperm contamination. It was further stored in sterile Eppendorf microcentrifuge tubes and mixed with an equal amount of sterilized physiological saline (0.85% NaCl). Precipitates present in the suspension were removed by centrifugation at 6000 rpm, and the supernatant was collected and stored at − 20 °C until analysis. The mucous portion was lyophilized, and its protein content was estimated according to Lowry, Rosenbrogh, Farr, and Randall (1951) using bovine serum albumin as a standard.

The lyophilized mucus was suspended in phosphate-buffered saline buffered (pH 7.4) at 1 mg/ml concentration to prepare the aqueous extract A. For the ethanolic extract preparation, the lyophilized sample (1 mg/ml) was suspended in absolute ethanol and centrifuged at 12,000 rpm for 45 min. The pellet was collected and washed with absolute ethanol for three more times, and the supernatant was discarded. The ethanol extracts were collected, suspended, and washed with distilled water to a final volume of 50 ml, and this was extracted with dichloromethane (CH_2Cl_2; 4 °C, 50 ml; extract B). The dichloromethane phase was collected and lyophilized; the finally dried extracts were dissolved in water and in 5% DMSO, respectively (Vennila et al., 2011).

Antimicrobial activity

Antibacterial activity of mucus extracts was determined by following Subramanian et al. (2007). The bacterial pathogens selected for the present study were *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Vibrio cholerae*, *Bacillus anthracis*, and *Klebsiella pneumoniae.* The fungal pathogens *Candida albicans*, *Aspergillus flavus*, *Mucor* sp., and *Trichoderma longibriachtin* were acquired from the Medical Microbiology Laboratory of Annamalai University. All eight species of bacterial strains were subcultured in Brain Heart Infusion Agar, and the fungal strains were maintained in Sabouraud Dextrose Agar. Antimicrobial activity of mucus extracts from *P. schlosseri* was determined by agar disc diffusion method against a 0.5 McFarland standard concentration of eight different bacterial strains and four fungal pathogens.

Sterile discs of Whatmann No. 1 filter paper were soaked with 30 μg/ml of each extract. The discs were placed on the surface of Mueller Hinton agar plates previously streaked with a broth culture of each pathogenic strain. The plates were incubated at 37 °C for pathogenic bacteria and 28 °C for pathogenic fungus. After the incubation, the diameter of the zone of incubation was measured using a caliper. Discs soaked with erythromycin (20 μg/disc) and nystatin (100 units/disc) were used as positive control for bacteria and fungi, respectively.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by serial dilution of the two extracts in four concentrations of 10, 20, 30, 40, and 50 μg/ml. Bacteria (2×10^8) were grown in Mueller Hinton broth incubated at 37 °C and fungi in Muller Hinton broth at 28 °C. MIC was determined as the lowest concentration necessary to inhibit the microbial growth. All assays were carried out in triplicate, using 2% DMSO as a negative control.

Hemolytic activity

Preparation of erythrocytes suspension

The mucus extracts were examined for the hemolytic activity against chicken and human erythrocytes. Fresh

chicken and human blood were mixed with 2.7% EDTA solution as anticoagulant and centrifuged at 5000 rpm for 10 min. Erythrocyte (3%) suspensions were used for hemolytic activity analysis (Caruso et al., 2014).

Microtitre plate assay

Hemolytic activity assay was performed in 96-well sterile Laxbro microtiter plates. Twofold serial dilutions of the mucus extracts were carried out in 100 μl of PBS (5 mg/ml), pH 7.4, and then, 100 μl of 3% red blood cell (RBC) suspension was added to each well. One hundred microliter of distilled water and 100 μl of PBS (pH 7.4) were added to the 3% RBC suspension for positive control and negative control, respectively. The plate was shaked in a gentle manner, and then, it was incubated for 2 h at room temperature. An evident red coloration in the wells was considered as a sign of positive hemolysis, while a pellet formation at the bottom of the wells was considered as a sign of a lack of hemolysis. The highest dilution of the extract resulting in a clear hemolysis was considered as the specific hemolytic titer.

Fourier-transform infrared spectroscopic analysis

The epidermal mucus extract A and B of giant mudskipper *P. schlosseri* were lyophilized to determine the functional group by using fourier-transform infrared (FT-IR) spectroscopy.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

In order to estimate the molecular weight of the active components of the proteinaceous gel, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the extracts was carried out in 10% polyacrylamide separating gels (*P. schlosseri* mucus protein). The gel was run in a BioRad electrophoresis apparatus for 1.5–5.5 h at 30 V during initial voltage then followed by 150 V until 2 mm from the base of the gel. The obtained bands were observed after silver staining (Manivasagan et al., 2009).

Results

The total protein concentration in the crude mucus extract of *P. schlosseri* was found to be 0.66 ± 0.04 mg/ml.

Antibacterial activity

The mucus extracts of *P. schlosseri* showed antibacterial activity against all eight clinical bacterial strains (Table 1). The mucus extract A showed the highest inhibition zone against *Ps. aeruginosa* (13 mm), whereas the lowest was observed against *S. aureus* (8 mm). The mucus extract B showed the highest diameter of inhibition zone against *S. typhi* (18 mm). The lowest inhibition zone (10 mm) was observed against *B. anthracis*.

Table 1 Antibacterial activity of mucus extract against human pathogens

S. No.	Name of the strains	Zone of inhibition (mm)		
		Standard	Aqueous extract	Organic extract
1	*Proteus mirabilis*	22	10	12
2	*Pseudomonas aeruginosa*	21	13	16
3	*Escherichia coli*	19	11	15
4	*Staphylococcus aureus*	17	08	12
5	*Salmonella typhi*	20	11	18
6	*Vibrio cholerae*	26	13	14
7	*Klebsiella pneumoniae*	18	11	12
8	*Bacillus anthracis*	17	09	10

Antifungal activity

The mucus extract of *P. schlosseri* showed antifungal activity against all four fungal strains tested (Table 2). The mucus extract A showed a maximum zone of inhibition (11 mm) against *C. albicans* and *T. longibriachtin*. The minimum zone of inhibition (9 mm) was observed against *A. flavus.* The mucus extract B showed higher zone of inhibition (16 mm) against *Mucor* sp., while the lower zone of inhibition (13 mm) was observed against *A. flavus*.

Minimum inhibitory concentration

The MIC values ranged between 3.0 and 25 mg/ml for the different bacterial pathogens; for the fungal pathogens, values ranged between 0.1 and 0.7 mg/ml. For both extracts (aqueous and dichloromethane phase), the lowest MIC value of bacterial pathogens (3 and 1 mg/ml) was found against *Ps. aeruginosa* and *K. pneumonia*, respectively. Regarding the fungal pathogens, both extracts showed a MIC of 0.7 mg/ml against *Mucor* sp. (Table 3).

Hemolytic activity

The mucus extracts A and B showed an hemolytic activity of 73.73 and 129.55 HT/mg against chicken

Table 2 Antifungal activity of mucus extract against the human pathogens

S. No.	Name of the strains	Zone of inhibition (mm)		
		Standard	Aqueous extract	Organic extract
1	*Aspergillus flavus*	18	09	13
2	*Mucor* sp.	22	10	16
3	*Candida albicans*	19	11	15
4	*Trichoderma longibriachtin*	16	11	14

Table 3 Minimum inhibitory concentration of mucus extracts

Microorganisms	Pathogens	Mucus extracts (mg/ml)	
		Aqueous extract	Organic extract
Bacteria	*Proteus mirabilis*	20	25
	Pseudomonas aeruginosa	03	12
	Escherichia coli	06	25
	Staphylococcus aureus	12	12
	Salmonella typhi	06	12
	Vibrio cholerae	08	05
	Klebsiella pneumoniae	12	01
	Bacillus anthracis	12	12
Fungi	*A. flavus*	0.8	0.9
	Mucor sp.	0.7	0.7
	C. albicans	1.2	1.1
	T. longibriachtin	1.3	1.0

erythrocytes and 43.47 and 73.73 HT/mg in A, B, AB, and O group of human blood, respectively (Tables 4 and 5).

FT-IR analysis

The FT-IR analysis of mucus extract A of *P. schlosseri* revealed the presence of different functional groups (Fig. 1). The analysis gave a sharp peak at 3428 cm^{-1}, which indicated the presence of O-H bounds in the mucus extract, depicting the presence of alcohols. Another sharp peak obtained in the extract was related to alkanes, with C-H bounds at 3026 cm^{-1}. The absorption peak at 1685 cm^{-1} was assigned to C=O bound vibration in carbonyl compounds. Moreover, the peak generated at 1438 cm^{-1} represented a CH_2 bending. The peak for the C-O stretch was at 1128 cm^{-1} and alkyl halide C-Cl stretch at 572 cm^{-1}.

The result of FT-IR analysis of mucus extract B gave a sharp peak at 3433 cm^{-1}, which indicated the presence of O-H bounds, a medium band at 3050 cm^{-1} for alkanes C-H bounds (Fig. 2), while peaks obtained at 1670 cm^{-1} showed the presence of C=O bounds related to carboxylic acids. Another peak at 1487 cm^{-1} represented a C-H_2 bending, C-O bounds at 1102 cm^{-1}, whereas alkyl halide C-Cl stretch at 615 cm^{-1}.

Table 4 Hemolytic activity of mucus extracts in chicken blood

S. No.	Sample	Source of blood—chicken blood		
		Total hemolysis (up to dilution)	Hemolytic unit (H/T)	Specific hemolytic activity (HT/mg)
1	Aqueous extract	4	8	73.73
2	Organic extract	5	16	129.55

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The SDS-PAGE analysis (Fig. 3) depicted the presence of evident protein bands of various molecular weights ranging from 14.3 kDa to 97.4 kDa. The mucus extract A showed bands at 97.4, 66, 43, and 29 kDa and the mucus extract B at 14.3, 29, and 43 kDa which is similar to the used standard.

Discussion

The involvement of epidermal mucus enzymes such as lysozyme, cathepsin B, and proteases in the innate immune mechanism of fish has been studied previously (Aranishi, 1999; Yano, 1996). Presence of certain hydrolytic enzymes including lysozyme, AP, cathepsin B, and proteases has been studied from a number of fishes, but no information is available for the fish species examined in this study.

In this study, the protein content in the crude mucus extract of *P. schlosseri* was found and similar reports also have been made by previous researchers; Manivasagan et al. (2009) reported the mucus of *A. maculates* have 12.64 μg/g of protein content; Wei, Xavier, and Marimuthu (2010) also reported protein content in both crude and aqueous mucus extract of *Channa striatus*. Dhotre, Bansode, and Shembekar (2013) also characterized the biochemical composition of freshwater fishes, viz., *Channa punctatus*, *Channa gachua*, *Cyprinus carpio*, and *A. dussmieri*, and found similar results.

In the present study, the *P. schlosseri* organic mucus extract (extracted with the polar solvent ethanol and non-polar solvents DMSO) exhibited greater potent inhibitory activity against both bacterial and fungal pathogens than the aqueous mucus extract against human pathogens tested. Earlier, Subramanian et al. (2008) also reported low microbial growth inhibition in aqueous fish mucus extracts of a wider range of fish species including Arctic char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), koi carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), haddock (*Melanogrammus aeglefinus*), and hagfish (*Myxine glutinosa*) could be due to the presence of low levels of enzymes. In contradiction, this strong inhibitory effect of aqueous mucus extract was reported from various eel fishes *Anguilla linnaeus* and *Mastacembelus armatus* (Bragadeeswaran & Thangaraj, 2011; Uthayakumar, Ramasubramanian, Senthilkumar, Brindha, & Harikrishnan, 2012). Hellio et al. (2002) reported that fish (*Pollachius virens*, *Labrus bergylta*, *Scophthalmus rhombus*, *Platichthys flesus*, *and Solea solea*) mucus extracted with ethanol and DMSO (organic extract) showed strong bactericidal activity against a broad range of pathogens. The results of the present study also indicated that the proteinaceous substances in the mucus

Table 5 Haemolytic activity of the different mucus extracts in human blood

S. No	Sample	Total hemolysis (up to dilution)	Hemolytic unit (H/T)	Specific hemolytic activity (HT/mg)—human blood			
				A	B	AB	O
1	Aqueous extract	4	8	41.0	36.1	43.47	43.47
4	Organic extract	5	16	ND	29.0	73.73	73.73

ND non-detectable

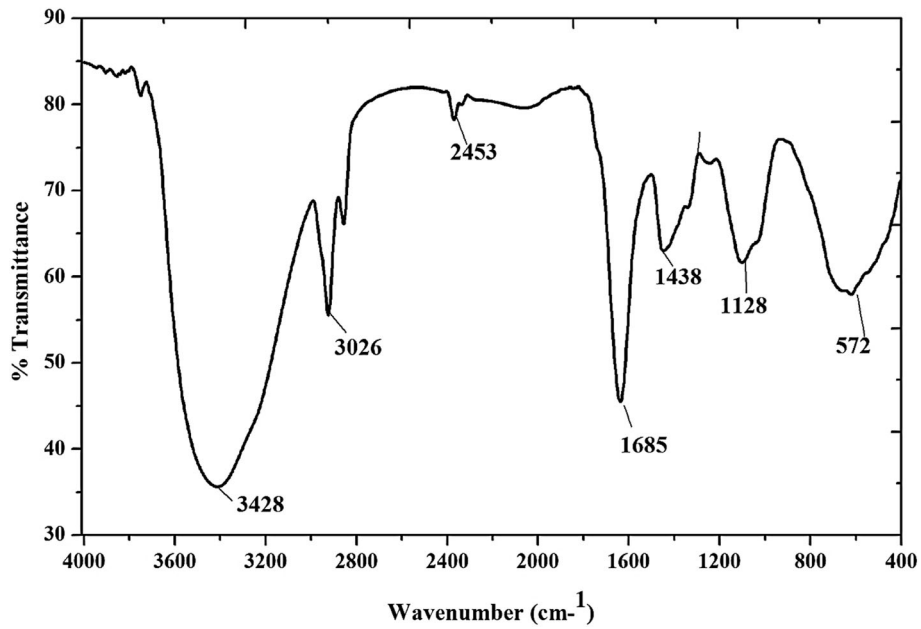

Fig. 1 Fourier-transform infrared spectrum analysis of *Periophthalmodon schlosseri* aqueous mucus extract

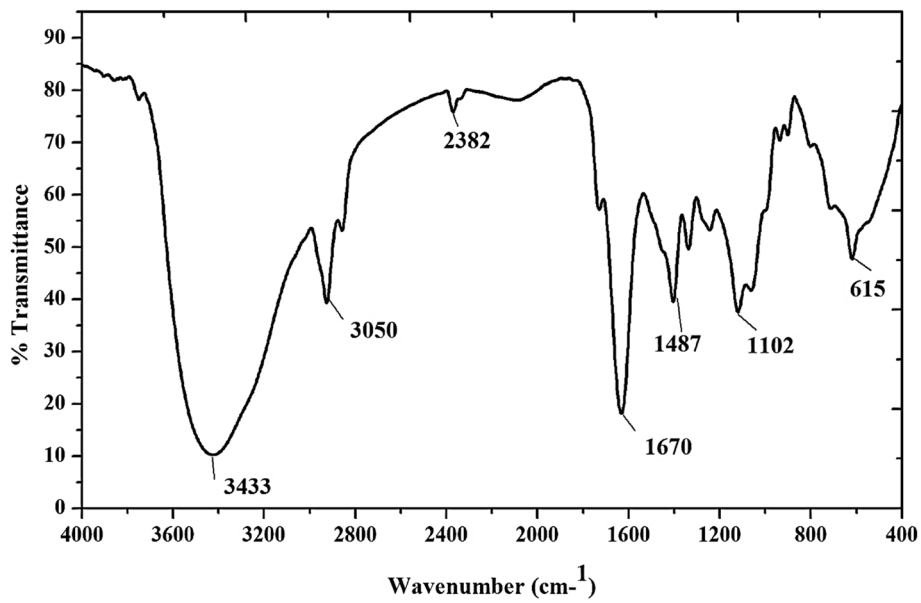

Fig. 2 Fourier-transform infrared spectrum analysis of *Periophthalmodon schlosseri* organic mucus extract

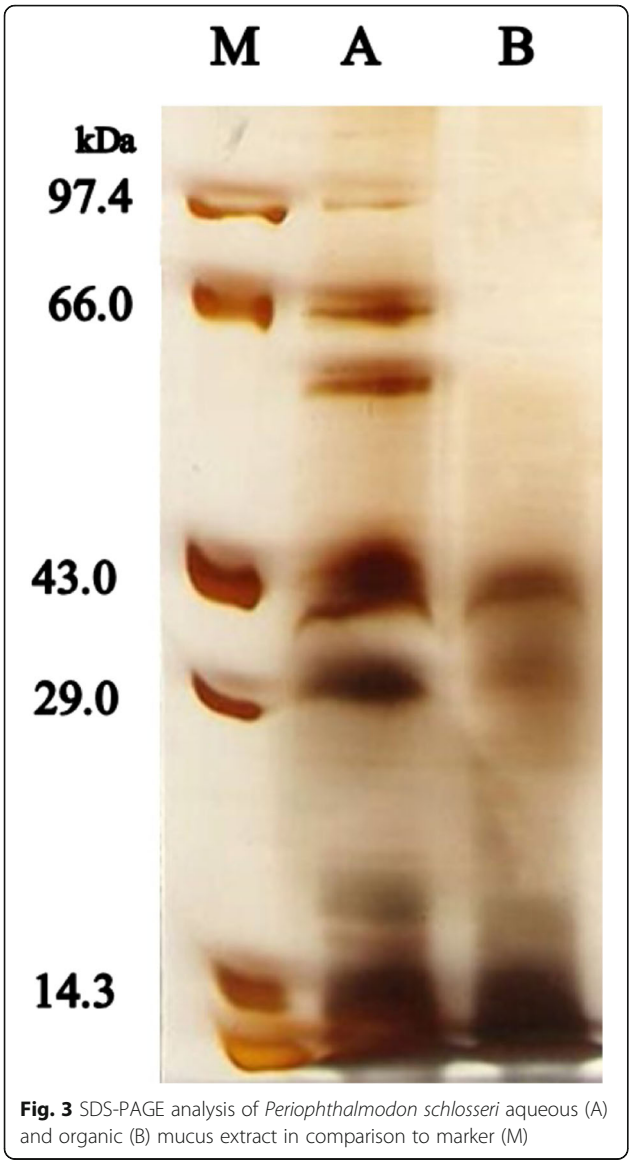

Fig. 3 SDS-PAGE analysis of *Periophthalmodon schlosseri* aqueous (A) and organic (B) mucus extract in comparison to marker (M)

of *P. schlosseri*, extracted using organic solvents, had most active antimicrobial components.

Further, the antimicrobial activity of epidermal mucus extracted with organic and aqueous solvents varies remarkably within and among the fish species (Subramanian et al., 2008). The variation in antimicrobial activities in the mucus of *P. schlosseri* 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.

The mucus of *P. schlosseri* contained protein derivatives which exhibited potent hemolytic activity when mixed with chicken and human blood. The mucus of *P. schlosseri* extracted using organic solvent (extract B) exhibited high level of hemolytic activity. Hellio et al. (2002) reported that lysozyme in the mucus has bacteriostatic properties and was ubiquitous in its distribution among living organisms. Bragadeeswaran, Priyadharshini, Prabhu, Raj, and Rani (2011) reported that the fish mucus of *Cynoglossus arel* and *Arius caelatus* has potential hemolytic activity. Similarly, Uthayakumar et al. (2012) also reported hemolytic activity (against sheep and cow blood cells) of mucus extracts of *M. armatus*.

The mucus of *P. schlosseri* extracted using organic solvent (extract B) showed low molecular mass protein bands (14.3, 29, 43.0 kDa) was prominent. The more intense protein bands of molecular mass 14.3 kDa were noticed in both mucus extracts. Rao et al. (2015) reported that the acidic extracts of *Chrysichthys nigrodigitatus* and *Tilapia* sp. showed more prominent bands in low molecular mass. Hence, this could be attributed that low molecular mass proteins of *P. schlosseri* mucus extracted using organic solvent could be a novel source of antimicrobial peptide. This study confirms that even the low concentration of organic extracts has high activity against both bacterial and fungal pathogens. The FT-IR analysis of the mucus of *P. schlosseri* showed wide spectral profile which confirms the presence of primary amine group, aromatic compound, halide group, aliphatic alkyl group, and polysaccharides.

Conclusions

There are many proteinaceous substances present in the fish mucus which exert strong resistance to invading pathogens. These findings prove that the mucus collected from the fish *P. schlosseri* shows strong antimicrobial and hemolytic potential. Further, purification of bioactive compounds is necessary in order to identify their chemical nature and to evaluate their potential as novel drugs.

Abbreviations

AMPs: Antimicrobial peptides; BSA: Bovine serum albumin; CH_2Cl_2: Dichloromethane; DMSO: Dimethyl sulfoxide; EDTA: Ethylenediaminetetraacetic Acid; FT-IR: Fourier-transform infrared spectroscopy; kDa: Kilodaltons; MIC: Minimum inhibitory concentration; MNPs: Marine natural products; PSA: Phosphate-buffered saline; RBC: Red blood cell; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Availability of data and materials

The data supporting the conclusions of this article is included within the article. The authors can be contacted for any additional supporting data required by the journal.

Authors' contributions
GM participated in the design of this research work and performed the collection of samples. VR contributed in the analytical part. KM undertook the characterization studies. GM, KM, and JV wrote the manuscript. VR supervised the findings of this work. All authors discussed the result and contributed to the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The authors declare that no animal was sacrificed for this study.

Consent for publication
Not applicable.

Competing interests
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