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Comparative assessment of organic solvent extraction on non-specific immune defences of skin mucus from freshwater fish

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Abstract

Fish skin mucus secretion is an important strategy against pathogens since it contains several immune molecules that act as the first line of defence. To date, several studies have reported that the mucus composition and immune responses vary depending on the fish species, and consequently, the comparative studies on skin mucus may have beneficial applications in the field of aquaculture. Therefore, the aim of the present study was to characterize functional groups of skin mucus collected from three different freshwater fish: common carp (*Cyprinus carpio*), rohu (*Labeo rohita*), and mrigal (*Cirrhinus mrigala*) and compare the antibacterial activity and innate immune parameters after organic solvent (acetone and methanol) extraction. Firstly, the Fourier transform infrared (FT-IR) spectral analysis of crude skin mucus demonstrated that the three fish species showed similar functional groups. Both the organic solvent extracts from skin mucus of three fish species exhibited antibacterial activity. Interestingly, skin mucus methanol extract from mrigal showed higher antibacterial activity when it was incubated with pathogenic bacteria tested and compared to the results found in the other fish skin mucus extracts. Regarding the innate immune-related enzymes, the lysozyme exhibited higher activity in the methanol extract of mrigal fish skin mucus compared to acetone extract as well as the other extracts of skin mucus from common carp and rohu fish. Alkaline phosphatase activity was significantly higher in skin mucus methanol extracts of common carp and mrigal fish compared to the acetone extract of the same species. In the case of protease enzyme, the activity observed was significantly higher in the skin mucus methanol extract compared to acetone extracts of all the three fish species, being the highest protease activity in the methanol extract of mrigal skin mucus. Therefore, our results demonstrated that the methanol extract of skin mucus displayed higher antibacterial and innate immune-related enzymes activities compared to acetone extract. When we compared to the species, the mrigal skin mucus extracts exhibited greater activities than common carp and rohu fish. These findings suggest that the methanol extract could be useful to isolate more bioactive molecules than the acetone extract in the species studied, which could be useful for therapeutic applications in aquaculture.

Extended author information available on the last page of the article

Keywords Fish skin mucus · Organic solvents (acetone and methanol) · Antibacterial activity · Innate immune-related enzymes · Freshwater species

Introduction

The aquatic ecosystem possesses numerous types of microorganisms than the terrestrial and the aquatic vertebrates survive against most of them by using innate immune system as a defence mechanism (Magnadóttir 2006). The skin mucus represents the evolutionary changes and adaptations of fish to the aquatic environment (Xu et al. 2013). Therefore, the skin mucus is an important component in fish innate immunity which acts as a semipermeable biological barrier between fish and the surrounding environment. The skin mucus is secreted by goblet cells and covers the entire epithelial surfaces (van der Marel et al. 2010). Sveen et al. (2017) indicated that the number of mucus cells reflects the mucosal tissues health status (Pittman et al. 2011) and the number of skin mucus cells increases in the presence of external stressors (intrinsic and extrinsic) such as hypoxia and high nitrate (Vatsos et al. 2010), exposure of aluminium (Ledy et al. 2003), lead (Dang et al. 2019), cadmium (Xie et al. 2019), and pathogens (van der Marel et al. 2010). Guardiola et al. (2015b) recorded increased immune enzyme activities in skin mucus of gilthead sea bream (*Sparus aurata*) upon heavy metal (arsenic, cadmium, and mercury) exposure. The skin mucus is also involved in several functions such as respiration, osmo-regulation, communication, locomotion, and disease resistance (Shephard 1994), as well as it plays an important role in mechanical and physiological protection (Kumari et al. 2011). In addition, the skin mucus allows the exchange of nutrients, water, and gases (Esteban 2012) and can be used to detect infections and environmental impacts on fish health (Reverter et al. 2018).

Fish skin mucus is composed of water, gel forming macromolecules including mucins, glycoproteins, complement proteins, C-reactive proteins, lectins, immunoglobulin molecules, antimicrobial peptides, and various biologically active substances as innate immune factors that kill invading pathogens (Ingram 1980; Sakai et al. 2001; Nigam et al. 2012; Sridhar et al. 2021a). It also contains large molecules such as egg yolk precursors (Meucci and Arukwe 2005) together with DNA and RNA material (Livia et al. 2006; Le Vin et al. 2011; Ren et al. 2015) and serves as a storage place for the elemental components such as sex steroids, corticosteroids, and stable isotopes (Shephard 1994; Church et al. 2009; Barkowski and Haukenes 2014; Guardiola et al. 2016). Recently, Liu et al. (2019) and Fuochi et al. (2017) reported the presence of heat shock proteins and chitinases in the skin mucus of mudskipper (*Boleophthalmus pectinirostris*) and common stingray (*Dasyatis pastinaca*). Moreover, the mucosal surface provides a niche for the non-pathogenic and the co-habitant microorganisms that can act as an additional barrier to pathogens through bacteriocins, antimicrobial peptides, H_2O_2, and other inhibitory compounds production (Cabillon and Lazado 2019). Therefore, the composition and the characteristics of skin mucus are important to maintain the immune functions of fish (Cone 2009).

The skin mucus is continuously produced and replaced to prevent the colonization of microbes (Nagashima et al. 2003). The secretion of skin mucus is higher in freshwater species than marine species due to exchange of water across fish skin (Shephard 1994; Tort et al. 2003). The skin mucus also serves as an important biomarker of stress (Tacchi et al. 2015; Guardiola et al. 2016) and helps to carry out minimally invasive environmental monitoring in fish (Ekman et al. 2015). The composition and antimicrobial activity of skin mucus vary among fish species (Esteban 2012; Guardiola et al. 2014a). For instance, freshwater fish striped snakehead (*Channa striatus*) skin mucus showed antibacterial activity against *Aeromonas hydrophila* (Wei et al. 2010). Rohu (*Labeo rohita*), catla (*Catla catla*), and mrigal (*Cirrhinus mrigala*) fish skin mucus exhibited different inhibition activity against *Aeromonas hydrophila*, *Pseudomonas fluorescens*, and *Edwardsiella tarda* (Dash et al. 2014). The marine teleost fish gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), shi drum (*Umbrina cirrosa*), common dentex (*Dentex dentex*), and dusky grouper (*Epinephelus marginatus*) skin mucus revealed bactericidal activity against pathogenic *Vibrio harveyi*, *Vibrio anguillarum*, *Photobacterium damselae*, and non-pathogenic bacteria *Escherichia coli*, *Bacillus subtilis*, and *Shewanella putrefaciens* (Guardiola et al. 2014a).

The immune response and bactericidal activity of fish skin mucus extracts have been demonstrated by several researchers in the recent years (Hellio et al. 2002; Subramanian et al. 2008; Bragadeeswaran et al. 2011; Nigam et al. 2012; Guardiola et al. 2015a; Al-Rasheed et al. 2018). Subramanian et al. (2008) indicated that the acidic skin mucus extracts of brook trout (*Salvelinus fontinalis*), haddock (*Melanogrammus aeglefinus*), and hagfish (*Myxine glutinosa*) showed higher bactericidal activity than that of the aqueous and the organic extract. Mahadevan et al. (2019) showed that the giant mudskipper (*Periophthalmodon schlosseri*) skin mucus organic extract displayed better inhibition activity than that of the aqueous extract against several bacterial and fungal pathogens.

Common carp (*Cyprinus carpio*) is distributed in all continents as a most potential aquatic species for aquaculture. Rohu (*Labeo rohita*) inhabits tropical freshwater of India and nearby nations, while mrigal (*Cirrhinus mrigala*) is an indigenous fish in the inland waters of India (Sridhar et al. 2021b). These fish species are considered as an important aquatic species of Indian aquaculture. Taking into consideration of the significance of the skin mucus in fish immunity, the purpose of this study was to compare the functional groups and immune-related enzymes which play a significant role in the innate immune mechanisms. Extraction of high efficiency of proteins using single organic solvent is quicker and simpler (Lin et al. 2007; Sridhar et al. 2021c). Want et al. (2006) mentioned that the organic solvent proved to be the most efficient technique for protein removal. Acetone and methanol solvents lead to softer protein pellets that could be easily solubilized (Santa et al. 2016). Therefore, this study was aimed to achieve a better understanding of the antimicrobial functions of the skin mucus against the pathogens after eliciting the proteins with acetone and methanol in the skin mucus of the three freshwater carp species with high commercial values. In addition, the efficacy of acetone and methanol solvents was also studied by comparing the irrespective of results. This scientific way of correlating the immunological properties of these fish species skin mucus extract would aid to acquire information about the bioactive molecules that could be used in the aquaculture health management.

Materials and methods

Fish collection and maintenance

The healthy live freshwater fish: common carp (*Cyprinus carpio*; 12.18 ± 1.06 cm and 62.88 ± 6.33 g), rohu (*Labeo rohita;* 8.35 ± 0.79 cm and 32.05 ± 2.67 g) and mrigal (*Cirrhinus mrigala*; 11.17 ± 1.28 cm and 24.44 ± 2.00 g) were collected from Nathan fish farm (Thanjavur, India) and stocked in freshwater circular tanks (2,000 L) in the Aquarium

Facility at Bharathidasan University (Tiruchirappalli, India). The water temperature was maintained at 30 ± 2 °C, pH at 7.36 ± 0.11, and the tanks were continuously aerated (0‰ salinity). The photoperiod was 12 h light: 12 h dark, and fish were fed with commercial pellet diet twice a day ad libitum. Fish were allowed to acclimatize for 15 days before the start of the experimental trial.

Skin mucus collection and extraction

The skin mucus was collected from three fish species ($n = 36$ combinedly) according to the method of Subramanian et al. (2008). The fish were starved for 1 day prior to skin mucus collection. To slough off the skin mucus, the fish were transferred into a sterile polyethylene bag containing 50 mM NaCl and gently moved back and forth. Then, the fish were returned to the recovery tanks. Skin mucus samples obtained from fish were pooled (3 pools of 4 fish each), centrifuged ($1500 \times g$, 10 min, 4 °C) to remove the insoluble particles, and the supernatants were collected and separated into three parts. The first part was lyophilized and stored at -20 °C. To the second part, four volumes of prechilled acetone were added to one volume of sample according to the modified protocol of Fic et al. (2010). Similarly, prechilled methanol was added in the same ratio into the third part. Organic solvents, acetone and methanol of HPLC grade, were used without additional purification. These mixtures were incubated overnight at – 20 °C, followed by centrifugation at $12{,}000 \times g$, 4 °C for 10 min. The resulting pellets were dissolved in 10 mM Tris–HCl buffer for further analysis. The protein concentrations of crude and organic solvent extracted skin mucus were determined by the method of Bradford (1976).

Fourier transform infrared spectral analysis

Fourier transform infrared (FT-IR) spectra were carried out for lyophilized fish skin mucus samples, and the functional groups were detected by potassium bromide (KBr) pellet technique. The dry lyophilized fish skin mucus sample of 10 mg was mixed with 100 mg of KBr, and it was compressed as a salt disc to read the spectrum in FT-IR spectrometer (Perkin Elmer, Norwalk, CT, USA). The spectra were recorded in the range of 400–4000 cm^{-1}.

Antibacterial activity

Clinical isolates of opportunistic fish pathogenic bacterium (*Aeromonas hydrophila*) and human pathogen (*Klebsiella pneumoniae*) were used to determine the antibacterial activity of acetone and methanol extracts of fish skin mucus samples. Bacteria were grown in nutrient agar (Sigma-Aldrich, Bengaluru, India) plates at 28 °C. Then fresh single colonies were diluted in 5 ml of nutrient broth (Sigma-Aldrich) and cultured for 24 h at 28 °C on an orbital incubator at 150–200 rpm. The antibacterial activity of fish skin mucus extracts was determined by using agar well diffusion method (Balouiri et al. 2016) with some modifications. Briefly, 100 µl of bacterial inoculum [1×10^7 CFU (colony forming unit) ml^{-1}] was spread on to the nutrient agar plates using sterile cotton swab. The wells with the diameter of 6 mm were punched in the agar plates, and 50 µl of each solvent extract of fish skin mucus was added on to the wells. Then, the plates were incubated at 37 °C overnight. The antibiotic streptomycin (10 µg ml^{-1}) and physiological saline (0.85% NaCl) were used as positive control and negative control, respectively. The antibacterial activity was determined in mm by measuring the diameter of the zone of inhibition around the well including the diameter of the well using Vernier caliper. Antibacterial activity data were the average of triplicates.

Innate immune parameters

Lysozyme activity

Lysozyme activity of skin mucus extracts collected from three different fish was determined by turbidometric method as described by Ross et al. (2000) using *Micrococcus lysodeikticus* cells. Briefly, the skin mucus extract samples were diluted 1/2 with 40 mM sodium phosphate (pH 6.2) buffer and then 50 µl of sample was transferred to a 96-well plate. The *Micrococcus lysodeikticus* cells (50 µl at the concentration of 0.3 mg ml^{-1} in 40 mM sodium phosphate buffer) were added to the mucus sample wells, and the absorbance was measured continuously at 450 nm for 50 min at 30 °C in a microplate reader (Synergy HT Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme that catalyzed a decrease in absorbance of 0.001 min^{-1}. The quantum of lysozyme present in skin mucus was obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma-Aldrich) through serial dilutions in the above buffer. Lysozyme values were expressed as U mg^{-1} mucus proteins.

Alkaline phosphatase activity

Alkaline phosphatase activity of skin mucus was determined by the method of Ross et al. (2000). Briefly, 50 µl of skin mucus extracts were prepared by reconstituting in 100 mM ammonium bicarbonate buffer with 1 mM $MgCl_2$ (pH 7.8, 30 °C) and transferred to a 96 well plate. Equal volume (50 µl) of 4 mM p-nitrophenol phosphate substrate was added to the wells containing skin mucus extracts. The absorbance was measured continuously at 405 nm for 30 min at 30 °C in a microplate reader (Synergy HT Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol product min^{-1}, and the activity was expressed as U mg^{-1} mucus proteins.

Protease activity

The skin mucus extract protease activity was analyzed as described previously by Ross et al. (2000) using azocaesin as substrate. Briefly, 50 µl of skin mucus extracts were suspended in 50 µl of 100 mM ammonium bicarbonate containing azocaesin substrate (0.35% w/v) and incubated in shaker at 30 °C for 19 h. The reaction was stopped by adding 100 µl of 5% trichloroacetic acid (TCA). Then, the tubes were centrifuged (15,400 × *g* for 5 min), and 100 µl of resulting supernatant was mixed with 100 µl of 0.5 N NaOH in a 96 well plate. The absorbance was measured at 450 nm using Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Skin mucus was replaced by trypsin (5 mg ml^{-1}, Sigma), as positive control (100% of protease activity), and by buffer, as negative control (0% activity). The percentage of trypsin activity compared to the positive control was calculated.

Statistical analyses

The experiments were conducted in triplicates, and the data were expressed as mean ± standard error of the mean (SEM). Then, the data were analyzed by two-way ANOVA (followed by Tukey tests) to determine the differences between the acetone and the methanol extracts for the same fish species and within each extracts regarding species, respectively. Normality of the data was previously assessed using a Shapiro–Wilk test, and the homogeneity of variance was also verified by using the Levene test. Non-normally distributed data were log-transformed to perform parametric tests, while non-parametric Kruskal–Wallis test followed by a Dunn's multiple comparison test was used when the data did not meet the parametric assumptions. All statistical analyses were conducted using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). The level of significance used was $P < 0.05$ for all the statistical tests.

Results and discussion

Fourier transform infrared (FT-IR) spectra

Fourier transform infrared (FT-IR) spectroscopic technique yields significant information regarding the functional groups present in the substance. The composition and structural elements of fresh or dried mucus can be obtained through FT-IR spectroscopy (Skingsley, 2010). FT-IR spectra of the lyophilized skin mucus of three fish species are depicted in Fig. 1. The results revealed the characteristic functional groups present in skin mucus of each fish species studied. Interestingly, similar patterns of peaks were observed for all the three fish skin mucus samples. The FT-IR spectrum of *C. carpio* skin mucus (Fig. 1a) showed a peak at 3428 cm^{-1} which indicated the presence of O–H bond. The peak at 1567 cm^{-1} showed the presence of carboxylate groups (O = C-O −). The peak at 1404 cm^{-1} was the characteristic of O–H bending of carboxylic acid. The peak at 1123 cm^{-1} was assigned to C-O stretching, and the peak at 695 cm^{-1} could be assigned to C–Br stretching. The similar functional groups were recorded by Kumar et al. (2019) in milkfish (*Chanos chanos*), Asian seabass (*Lates calcarifer*), and grey mullet (*Mugil cephalus*) skin mucus. Manikantan et al. (2016) reported the presence of sulfones group based on the FT-IR absorption peaks obtained at 1120–1170 cm^{-1} in the organic skin mucus extract of greasy grouper (*Epinephelus tauvina*).

The FT-IR result of *L. rohita* skin mucus (Fig. 1b) showed a peak at 3429 cm^{-1} indicating the presence of O–H bond. The peak obtained at 1567 cm^{-1} indicated the presence of carboxylate group (O = C-O −). Another peak at 1404 cm^{-1} represented O–H bending of carboxylic acid. The peak obtained at 1123 cm^{-1} showed the presence of C-O stretching. The peak at 882 cm^{-1} was assigned to C-H bending, and the peak at 652 cm^{-1} represented C–Br stretching. Similar FT-IR spectrum along with C–Cl stretch was observed by Mahadevan et al. (2019) in the skin mucus extract of giant mudskipper (*Periophthalmodon schlosseri*). FT-IR spectra of large scale tonguesole (*Cynoglossus arel*) and sea catfish (*Arius caelatus*) skin mucus revealed the presence of alkyl amine and/or cyclic amine with polysaccharides (Bragadeeswaran et al. 2011).

The FT-IR spectrum of the *C. mrigala* fish skin mucus sample (Fig. 1c) showed a characteristic peak at 3428 cm^{-1} which corresponded to O–H bond. The absorption

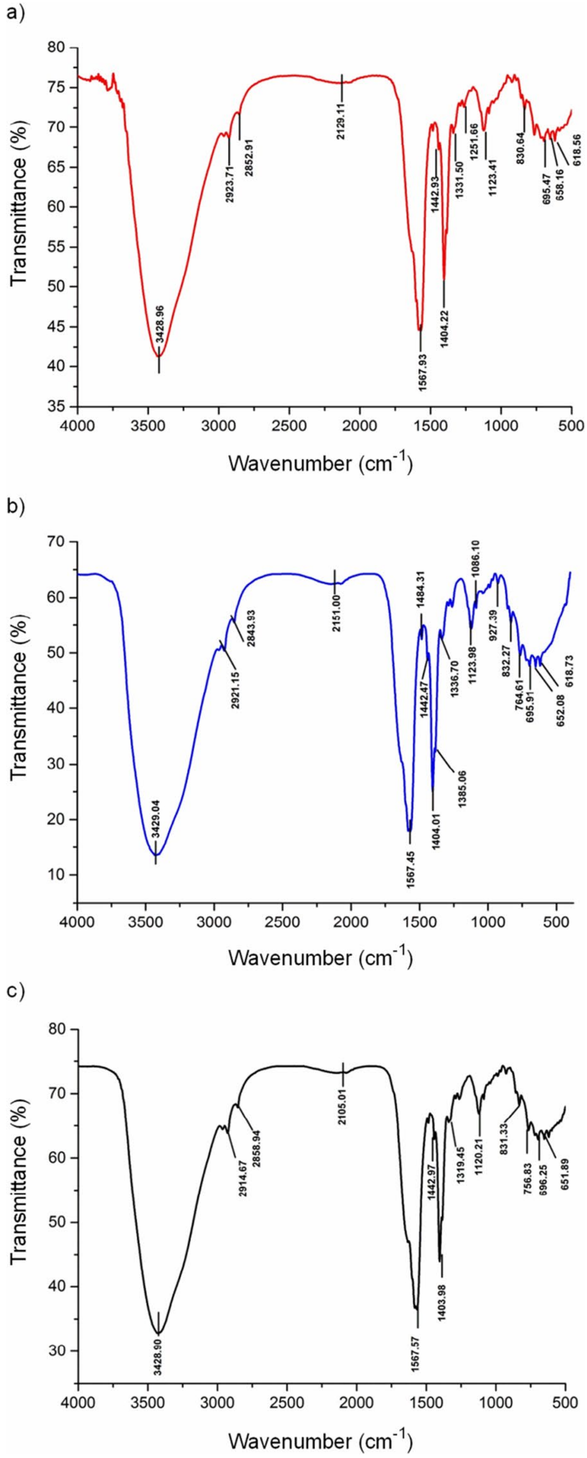

Fig. 1 FT-IR spectral analysis of crude skin mucus of freshwater fish common carp (*Cyprinus carpio*) (**a**), rohu (*Labeo rohita*) (**b**), and mrigal (*Cirrhinus mrigala*) (**c**)

peak at 1567 cm^{-1} was assigned to O = C-O – (carboxylate group). A peak at 1404 cm^{-1} indicated O–H bending of carboxylic acid. The peak for C-O stretch corresponded at 1128 cm^{-1}, and C-H bending was at 831 cm^{-1}. The peak at 696 cm^{-1} indicated the presence of C–Br stretching. The present results were in agreement with Waghmare et al. (2014), who observed three distinct peaks for Nile tilapia (*Oreochromis niloticus*) skin mucus representing alcohols and phenols. After analyzing the skin mucus of three brackish water fish milkfish (*C. chanos*), Asian seabass (*L. calcarifer*), and grey mullet (*M. cephalus*) in FT-IR, Kumar et al. (2019) indicated that the functional groups, viz., aldehyde, alkene, and isothiocyanate, were commonly found in all the three brackish water fish skin mucus. Moreover, in this study, the FT-IR spectra of *C. carpio*, *L. rohita*, and *C. mrigala* skin mucus revealed the presence of alcohols, cyclic alkenes, and carboxylic acids. Changes in the FT-IR absorption bands clearly indicate the alterations in the composition (Titus et al. 2019). Since the fishes studied in this experiment were from the same species, the FT-IR spectra also share identical peaks revealing their similar composition of skin mucus.

Antibacterial activity

The acetone and methanol skin mucus extracts of three fish species showed antibacterial activity against *A. hydrophila* and *K. pneumoniae* (Fig. 2). In the case of *A. hydrophila*, no significant variations were observed between acetone and methanol solvents in each fish species except *C. mrigala* where the methanol extract displayed significant antibacterial activity compared to acetone (Fig. 2a). When *K. pneumoniae* was incubated with the extracts, no significant variations in activity were observed between acetone and methanol in *C. carpio*, but higher activity was observed in the methanol extract of *L. rohita* and *C. mrigala* fish skin mucus with respect to acetone extract (Fig. 2b). The disparity in the antibacterial activity of fish skin mucus observed could be ascribed to the characteristic features of mucus secreted in each fish species (Subramanian et al. 2008). In this study, variations in the antibacterial activity of skin mucus observed against similar or different microbes might be the reason of differences in the quality and quantity of proteins and enzymes present in skin mucus of each fish species as suggested by Balasubramanian et al. (2012). In addition, levels of alkaline phosphatase, protease, cathepsin B, and lysozyme also influence the antibacterial activity of fish skin mucus (Subramanian et al. 2007; Esteban 2012; Mansouri Taee et al. 2017).

Among the solvent extracts of three fish species, significant inhibition activity was observed in the methanol extract of mrigal fish against *A. hydrophila* and *K. pneumoniae* when compared with other fish skin mucus extracts. In this way, Guardiola et al. (2015a) observed that the organic skin mucus extract of European sea bass (*Dicentrarchus labrax*) affected the growth of several fish pathogenic bacteria (*Vibrio harveyi*, *Photobacterium damselae*, *V. anguillarum*, and *Shewanella putrefaciens*) ranging from 43 to 70% depending on the bacterial species. In another study, the organic extract of giant mudskipper (*Periophthalmodon schlosseri*) skin mucus displayed better antibacterial activity than aqueous extract against human bacterial pathogens (*Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *V. cholerae*, *Bacillus anthracis*, and *K. pneumoniae*) and fungal strains (*Candida albicans*, *Aspergillus flavus*, *Mucor* sp., and *Trichoderma longibriachtin*) (Mahadevan et al. 2019).

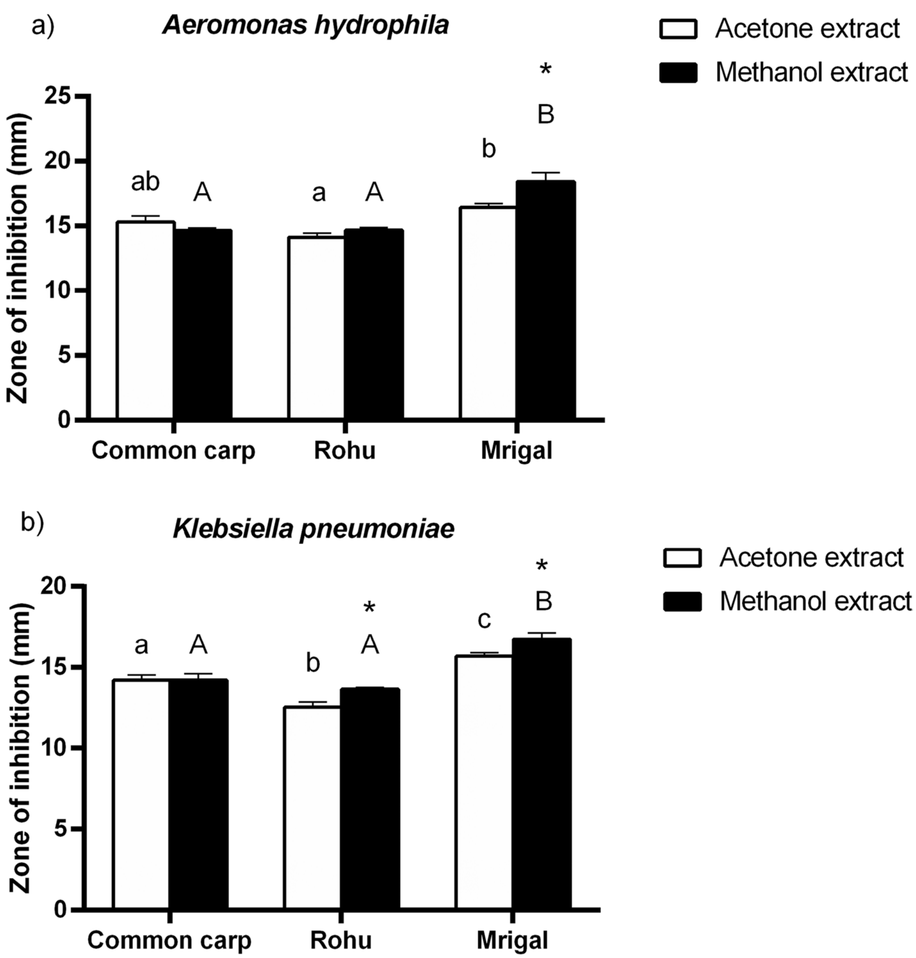

Fig. 2 Antibacterial activity in acetone and methanol extracts of skin mucus from freshwater fish common carp (*Cyprinus carpio*), rohu (*Labeo rohita*), and mrigal (*Cirrhinus mrigala*) against *Aeromonas hydrophila* (**a**) and *Klebsiella pneumoniae* (**b**). Bars represent the mean ± SEM. Asterisks denote significant differences between acetone and methanol extracts for the same species whilst small and capital letters denote significant variations within each extracts regarding species (two-way ANOVA; $P < 0.05$)

Innate immune parameters

The protein concentrations present in the skin mucus of *C. carpio* were 3.397 ± 0.168, 1.088 ± 0.060, and 0.973 ± 0.045 mg ml^{-1}; *L. rohita* were 2.242 ± 0.103, 0.733 ± 0.022, and 0.680 ± 0.034 mg ml^{-1}; and *C. mrigala* were 2.718 ± 0.045, 0.893 ± 0.059, and 0.767 ± 0.036 mg ml^{-1} for crude, acetone, and methanol extraction, respectively. The lysozyme, alkaline phosphatase, and protease activities revealed variations in the extracts of three fish species skin mucus. Lysozyme levels are considered as an important index of the innate immunity in fish and known as an antibacterial enzyme (Ellis 2001). The lysozyme activates the complement system and phagocytes (Paulsen et al. 2003; Gonzalez-Silvera et al. 2018). In the present study, the lysozyme activity was higher in the acetone extract of *C. carpio* and *L. rohita* compared to the methanol extract. However, the methanol extract of *C. mrigala* showed higher lysozyme activity with respect to the acetone extract (Fig. 3a). Interestingly, the level of lysozyme activity was greater in the methanol extract from skin mucus of *C. mrigala* respect to the values found in the other methanol extract of *C. carpio* and *L. rohita* skin mucus. A low level of lysozyme activity was also reported in *L. rohita* skin mucus by Nigam et al. (2012) when compared with the other freshwater teleosts fish *C. mrigala*, *C. catla*, spotted snakehead (*Channa punctatus*), and bagrid catfish (*Rita rita*) skin mucus. The reduced skin mucus lysozyme activity was reported in *C. mrigala* than *L. rohita* by Dash et al. (2014) and indicated that the discrepanciese in lysozyme activity were also attributed to the factors such as sex, diet, maturity, and handling stress. In this study, variations in skin mucus lysozyme activity were observed on account of fish species and solvents used. The lysozyme activity of the Atlantic salmon (*Salmo salar*) and *C. carpio* skin mucus changed in stressed condition and different diets, respectively (Balfry and Iwama 2004; Hoseinifar et al. 2016).

Alkaline phosphatase, which is a lysosomal enzyme, acts as an antibacterial agent based on its hydrolytic activity. In the recent past, several authors have reported that alkaline phosphatase protects during infections and helps in skin regeneration in fish (Subramanian et al. 2007; Palaksha et al. 2008; Roosta and Hoseinifar 2016). Guardiola et al. (2014b) demonstrated the presence of alkaline phosphatase in the skin mucus of *S. aurata* and revealed stronger bactericidal activity. Though the accurate function of alkaline phosphatase is yet to be explained, this enzyme exhibited antimicrobial activities in skin mucus as an epithelial anti-inflammatory mechanism that detoxifies pro-inflammatory microbial compounds and suggested alkaline phosphatase is a component of innate immunity in fish that provide protection against microbes (Lallès 2019). Current results showed that the alkaline phosphatase activity (Fig. 3b) was higher in the methanol extract of *C. carpio* and *C. mrigala* skin mucus than the acetone extract. However, when we compare all the three fish skin mucus acetone and methanol extracts, alkaline phosphatase activity observed was higher in the methanol extract of *L. rohita* and *C. mrigala.* The methanol extract of three fish skin mucus showed higher activity of alkaline phosphatase than that of the acetone extract. Activity of alkaline phosphatase modifies depending on stress, season, and infections (Jung et al. 2012). Dash et al. (2014) observed higher alkaline phosphatase activity in *C. mrigala* and lower activity in *L. rohita* after comparing the innate immune parameters of skin mucus from three Indian major carps: *L. rohita*, *C. catla*, and *C. mrigala*. On account of growth, physiological and ecological conditions, the activity of alkaline phosphatase in freshwater fish skin mucus differ (Kumari et al. 2019). Likewise, in this study, differences in the alkaline phosphatase activity were observed among the fish species with respect to the solvents. In another study, Subramanian et al. (2007) reported the highest activity of alkaline phosphatase in the skin mucus crude extract of koi carp (*C. carpio*) compared with the other freshwater fish Arctic char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), and striped bass (*Morone saxatilis*).

Other enzyme studied was the protease which plays a protective role in fish skin mucus against pathogens as a natural resistance (Ingram 1980) by cleaving the proteins of microbes and hinders the process of colonization and invasion (Guardiola et al. 2014b). Many proteases have been characterized in various fish skin mucus (Dash et al. 2018). In the present study, the methanol extract of all the three fish species skin mucus showed higher protease activity in comparison with the values obtained in the acetone extract (Fig. 3c). Interestingly, these results are in agreement with the activity of lysozyme and alkaline phosphatase. The methanol extract of skin mucus from *C. mrigala* recorded a significant higher protease activity compared to the other fish skin mucus solvents extracts. These results are supported by the findings of Nigam et al. (2012), who reported higher

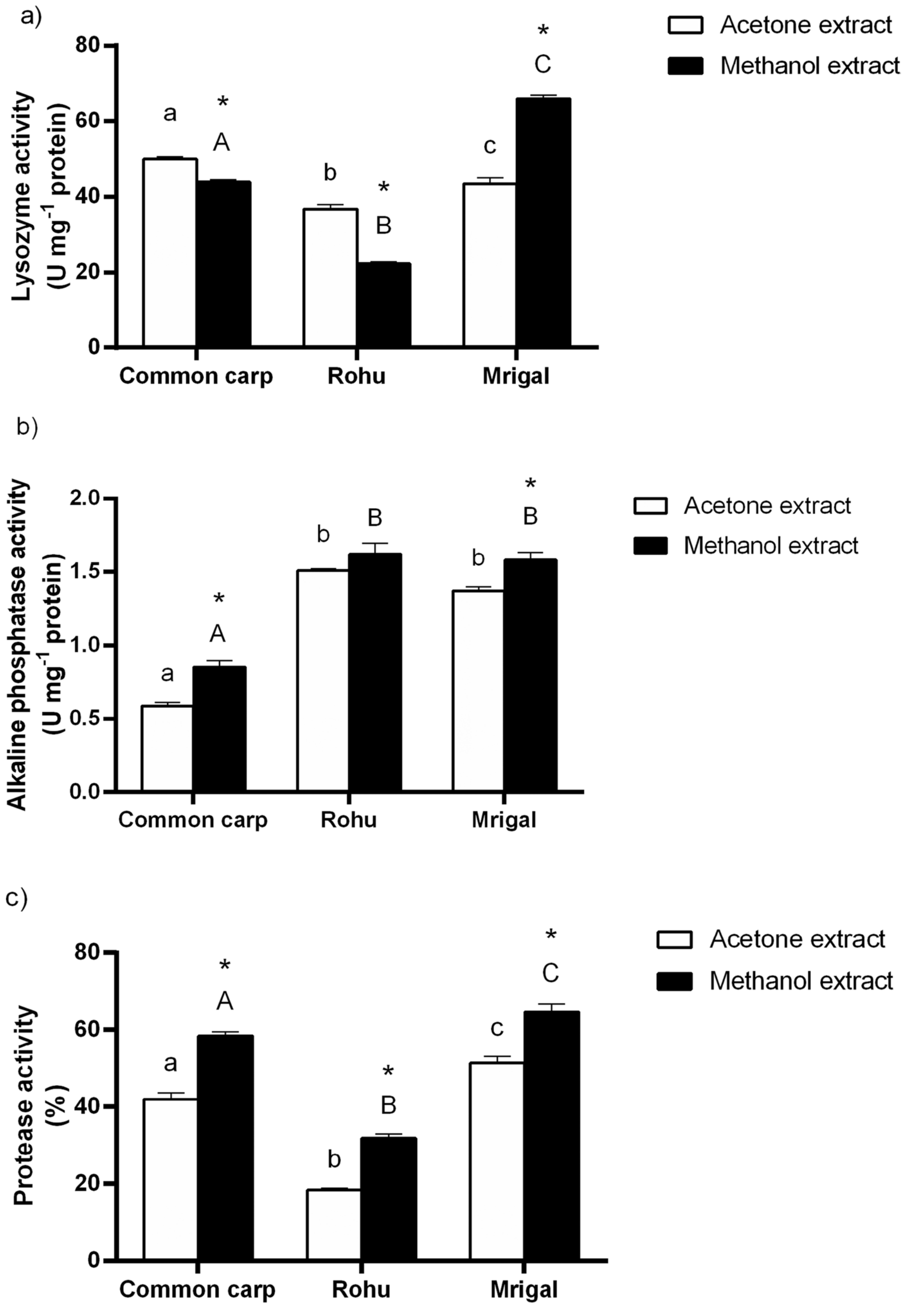

Fig. 3 Lysozyme (U mg^{-1} protein) (**a**), alkaline phosphatase (U mg^{-1} protein) (**b**), and protease (%) (**c**) activities in acetone and methanol extracts of skin mucus from freshwater fish common carp (*Cyprinus carpio*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*). Bars represent the mean ± SEM. Asterisks denote significant differences between acetone and methanol extracts for the same species whilst small and capital letters denote significant variations within each extracts regarding species (two-way ANOVA; $P < 0.05$)

activity of protease in the skin mucus of *C. punctatus* and *C. mrigala*. However, the results of the present study are not in agreement with Dash et al. (2014), who indicated the higher protease level in the skin mucus of *L. rohita* and the lower level in *C. mrigala*. In this study, the differences in the protease activity among the fish species might be owing to the presence of differential proteins between the species and the organic solvent skin mucus extracts. In addition, the levels of protease in fish skin mucus vary depending on the stressors (Guardiola et al. 2016; Sanahuja et al. 2019).

Adopting protein extraction methods ranges from organic solvents to acids for profiling. Generally, the organic solvent primarily ascribing two reasons to extract proteins, i.e., decreases in protein solubility resulting from reduction of the dielectric properties of the solution and destruction of the membrane protein hydration (Cowan 1997). It displaces the water molecules around the hydrophobic regions on the protein surface, and as a result of surrounding organic solvent, hydrophobic interactions between proteins are reduced and lead to aggregation of proteins (Bueno et al. 2011). Additional advantage of organic solvent extraction is the rapid concentration of proteins (Verostek et al. 2000). Most of the hydrophilic proteins are extracted by acetone, while methanol extracts hydrophobic proteins (Arruda et al. 2019).

The methanol extract showed better result than acetone in this study might be due to the reason of acetone, which has the ability to interact with non-polar regions of proteins and reduces its activity (Mohammad-Beigi et al. 2016). In addition, acetone is more difficult in microscale due to its efficiency and the loss of proteins (Crowell et al. 2013). Acetone has higher dielectric constant than methanol and lower viscosity. These parameters change the dissociation forces and cause different protein extraction mechanism (Alzweiri et al. 2008). The recovery of Chinese hamster (*Cricetulus griseus*) ovary host cell protein was done using different solvents and showed that methanol solvent recovered more proteins than acetone and TCA (Valente et al. 2014). Current study results revealed that the methanol precipitation followed by centrifugation seems to be the most effective approach for proteins as already mentioned by Want et al. (2006). The extracellular lipase from *Pseudomonas monteilii* TKU009 retained 80% of its activity after extracting with methanol than acetone, ethanol, and isopropanol which retained 50–75% of lipase activity (Wang et al. 2009). Afkarian et al. (2010) identified that during the discovery of biomarker in urine, the methanol precipitation method yielded high protein levels than ethanol and acetonitrile. Similarly, after extracting proteins with solvents such as acetonitrile, perchloric acid, and TCA, methanol solvent was the best choice for protein extraction (Gowda and Raftery 2014). The divergence found in the enzymatic activity of fish skin mucus in this study could be attributed to the habitat, evolutionary adaptation to environmental nutritional factors (Lallès 2019; Wang et al. 2019), and phylogenetic divergence (Nigam et al. 2012). This study demonstrated that the methanol extract of *C. mrigala* skin mucus showed significantly higher activity in antibacterial and enzymatic analysis when compared with the acetone and methanol solvents of *L. rohita* and *C. carpio* fish skin mucus.

Conclusion

The results of the present study suggested that organic solvents, especially the methanol, which was used for the extraction of proteins from skin mucus of *C. carpio*, *L. rohita*, and *C. mrigala* aided in achieving better results in the antibacterial activity and in the innate immune-related parameters tested. Interestingly, the methanol extracted skin mucus from

C. mrigala showed higher lysozyme and protease activities and displayed significant antibacterial activity against the bacteria assayed. However, it exhibited similar activity level with *L. rohita* independently of the type of extract in the case of alkaline phosphatase. Therefore, further investigation on the bioactive molecules from methanol extracted fish skin mucus of mrigal fish would be useful in aquaculture to combat fish pathogens.

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Data Availability All data generated or analyzed during this study are included in this published article.

Code availability Not applicable.

Declarations

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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