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Antimicrobial and hemolytic activity of fish epidermal mucus *Cynoglossus arel* and *Arius caelatus*

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ABSTRACT

Objective: To study the antimicrobial, hemolytic activity and immunomodulatory activity of fish epidermal mucus and their chemical constituents from *Cynoglossus arel (C. arel)* and *Arius caelatus (A. caelatus)*. Mucus plays an important role in the prevention of colonization by parasites, bacteria and fungi. **Methods:** Epidermal mucus was obtained from two marine fishes, lyophilized and the chemical composition of epidermal mucus was analysed by FT-IR analysis. The *in vitro* antimicrobial activity against human pathogens (fungi, gram positive and gram-negative bacteria) and also the hemolytic activity and immunomodulatory activity were determined. **Results:** Totally ten human pathogens were tested against the fish mucus. Out of the ten pathogens, five pathogens have proved to be sensitive to the mucus. Maximum zone of inhibition was observed against *Vibrio cholera (V. cholera)* (9 mm and 2 mm in diameter), followed by *Staphylococcus aureus (S. aureus)* with a inhibition zone of (6 mm and 3 mm), *Streptococcus areus (S. areus)* (5 mm and 4 mm), *Vibrio parahemolyticus (V. parahemolyticus)* (4 mm and 5 mm) respectively. **Conclusions:** The present investigation has revealed that positive progresses in the fish mucus extracts against human pathogens and hemolytic activity. But further efforts are required for the purification and isolation of the active antimicrobial compounds in order to establish their possible applications.

1. Introduction

Fish by-products are rich in potentially valuable proteins, minerals, enzymes, pigments or flavours[1]. Use of skin fish or mucus for research on biologically active compounds could be an interesting exercise. The biological interface between fish and their aqueous environment consists of a mucus layer composed of biochemically diverse secretions from epidermal and epithelial cells[2]. The mucus layer covers the surface of external body to reduce body friction against water and to protect from abrasion injury. It's have a variety of biologically active substances in the mucus significantly act as humoral defense factors, since the fish immunity is less sophisticated than that of higher animals[3,4]. Particularly the antimicrobial agent appears to play an important role in aquatic organisms including fishes, which are always expressed to pathogenic microorganisms through the surrounding water. Over the past years, fish mucus has also been plays a role in the prevention of colonization by parasites, bacteria and fungi[5,6]. The antibacterial role of fish mucus has been known for many years but previous works on antibacterial tests has been directed towards marine microbial strains. It was reported that epithelial tissues produce antimicrobial molecules which serve as the first line of a host's defense against microbial invasion in a variety of vertebrates including humans[7]. In this present study, a series of solvent extracts of mucus from two marine fishes were screened for their *in vitro* activity against terrestrial pathogens (fungi, gram positive and gram-negative bacteria) and also to determine the hemolytic activity and FT-IR analysis of the mucus.

2. Materials and methods

2.1. Collection of mucus from fish

Specimens of *Cynoglossus arel (C. arel)* and *Arius caelatus (A. caelatus)* were collected at Pazhiyyar landing center, (Lat 11° 21′32.27″ N and long 79° 49′24.92″ E) (Figure 1)

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transported to the laboratory and stored at −20 ℃.

Figure 1. Location of the sampling site.

2.2. Preparation of skin mucus extract

After frozen specimens were partially thawed and the mucus was carefully scraped from the dorsal body using a sterile spatula Al-Hassan *et al*[8]. Mucus was not collected in the ventral side to avoid intestinal and sperm contamination. The mucus samples were collected aseptically from the fish and thoroughly mixed with equal quantity of sterilized physiological saline (0.85% NaCl) for the antimicrobial studies. The precipitate in suspension was removed by centrifugation at 6 000 × *g*. The supernatant was then collected and lyophilised. This extract was stored at 4 ℃ for further use.

2.3. Microbial strains used

Antimicrobial activity of fish mucus was determined against 10 bacterial strains viz., *Escherichia coli (E. coli)*, *Klebsiella oxytoca (K. oxytoca)*, *Klebsiella pneumoniae(K. pneumoniae)*, *Lactobacillus vulgaris (L. vulgaris)*, *Proteus mirabilis (P. mirabilis)*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Salmonella typhi (Salmonella typhi)*, *Salmonella paratyphi (S. paratyphi)*, *Staphylococcus aureus (S. aureus)* and *Vibrio cholera (V. cholera)* and ten fungal strains *Aspergillus niger (A. niger)*, *Candida albicans (C. albicans)*, *Aspergillus flavus (A. flavus)* *Mucor* sp., *Alternaria alternata (A. alternata)*, *Pencillium* sp., *Rhizopus*, *Trichophyton rubrum (T. rubrum)*, *Trichophyton mentagarophytes (T. mentagarophytes)*, *Epidermophyton floccosum (E. floccosum)*. All microbial strains were obtained from the Department of Medical Microbiology, Rajah Muthiah Medical College, Annamalai University, India.

2.4. Anti microbial assay

The spectrum of antimicrobial activity was described by Bauer *et al*[9]. Antimicrobial activity was expressed in terms of diameter of zone of inhibition were measured in mm by using Vernier caliper or a scale and recorded.

2.5. Determination of hemolytic assay and protein

The crude mucus extracts of 11 fractions were assayed for their hemolytic activities on chicken and goat erythrocytes. Chicken and goat blood were obtained from a nearby slaughterhouse, using 2.7% ethylenediaminetetraacetic acid (EDTA) solution as an anticoagulant at 5% of the blood volume, and brought to the laboratory. The blood was centrifuged thrice at 5 000 rpm for 5 min; 1% erythrocyte suspension was prepared by adding 99 mL normal saline to 1 mL of packed erythrocytes. The micro hemolytic assay was performed in 96-well 'v' bottom microtitre plates[10]. Serial two-fold dilutions of the crude mucus were made in 100 μL of normal saline. Then 100 μL of 1% erythrocyte was added to all the wells. For positive control, 100 μL of distilled water and for negative control 100 μL of normal saline were added respectively to the 1% red blood cell (RBC) suspension. The plate was gently shaken and allowed to stand for two hours at room temperature. The presence of uniform red color suspension in the wells was considered to be positive hemolysis and a button formation in the bottom of the wells constituted a lack of hemolysis. The reciprocal of the highest dilution of the crude toxin showing the hemolytic pattern (hemolytic unit) was divided by the protein content to obtain the specific hemolytic unit and the protein was estimated by the method of Lowry *et al*[11].

2.6. Fourier transform – infra red spectrum analysis

FT-IR spectroscopy of solid samples of mucus from two marine fishes relied on a Bio-Rad FTIR-40 model, USA. Sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed further to prepare as a salt disc (10 mm diameter) for reading the spectrum[12].

2.7. Immunomodulatory activity

2.7.1. Neutrophil locomotion and chemotaxis test

Neutrophil cell suspension was prepared in phosphate buffer saline solution (PBS) at about 106 cells/mL. The lower compartment of chemo tactic chamber to a pH 7.2 *eg*. chamber 1-PBS solution (control); chamber 2-casein 1 mg/L (standard); and chamber 3, 4, 5, 6, 7 with different concentrations (10, 25, 50, 100 and 1 000 μg/mL) of test sample. The upper compartment (1 mL syringe) was filled with neutrophil cell suspension and the wet filter (Millipore) 3 mm pore size was fixed at the bottom of the upper compartment. The upper compartment was placed into the lower compartment and incubated at 37 ℃ for 180 min.

The upper compartment was removed and inverted to empty the fluid. The lower surface of the filter was fixed with 70% ethanol for 2 min and then stained with Haematoxylin dye for 5 min. The fixed filters were observed under microscope using 100× lenses and number of neutrophil cells reached to the lower surface was counted.

2.7.2. Preparation of C. albicans suspension

The *C. albicans* culture was incubated in Sabouraud broth overnight and then centrifuged to form a cell bottom and supernatant was discarded. The cell button washed with sterile Hank's balanced salt solution (HBSS) and centrifuged again. This was done 3-4 times. The final cell button was mixed with a mixture of sterile HBSS and human serum in proportion of 4:1. The final cell suspension of concentration 1×10^8 was used for the experiment.

2.7.3. Slide preparation

Human blood (0.2 mL) was obtained by finger prick method

on sterile glass slide and incubated at 37 ℃ for 25 min to allow clotting. The blood clot was removed very gently and slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophils (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flood with concentration of test sample and incubated at 37 ℃ for 15 min. The PMNs were covered with *C. albicans* suspension and incubated at 37 ℃ for 1 h. The slide was drained, fixed with methanol and stained with Giemsa stain.

2.7.4. Phagocytosis evaluation

The mean number of *Candida* cells phagocytosed by PMNs on the slide was determined microscopically for 100 granulocytes using morphological criteria. This number was taken as phagocytic index (PI) and was compared with basal PI of control. This procedure was repeated for different concentrations (10, 25, 50, 10 and 1 000 μg/mL) of test sample. Immunostimulation in % was calculated by using following equation:

Stimulation (%) = PI (test)—PI (control) ×100/PI (control)

2.7.5. Qualitative nitroblue tetrazolium chemotaxis test[13]

A suspension of leucocytes (5×10^6 /mL) was prepared in 0.5 mL of PBS solution in 7 tubes. 0.1 mL PBS solution (control) and 0.1 mL of endotoxin activated plasma (standard) is added to 1st and 2nd tube respectively and to the other 4 tubes added 0.1 mL of different concentrations (10, 25, 50, 100 and 1 000 μg/mL) of the test samples; 0.2 mL of freshly prepared 0.15% NBT solution was added to each tube and incubated at 37 ℃ for 20 min. Centrifuged at 400 g for 3–4 min to discard the supernatant.

The cells were resuspended in the small volume of PBS solution. A thin film was made with the drop on the slide, dried, fixed by heating, counterstained with dilute carbol–fuchsin for the 15 sec. The slide was washed under tap water, dried and focused under 100× oil immersion objective; 200 neutrophils were counted for the percentage of NBT positive cells containing blue granules/lumps.

2.8. Statistical analysis

The data were analysed using one–way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. *P* values <0.05 were considered significant.

3. Results

3.1. Antimicrobial assay

The antibacterial activity of mucus of the *C. arel* and *A. caelatus* are presented in the Figure 2. The mucus collected from marine fishes shows a strong inhibition in the growth of tested bacteria. Maximum zone of inhibition was observed against *V. cholera* (9 mm and 2 mm in diameter), followed by *S. aureus* with a inhibition zone of (6 mm and 3 mm), *S. areus* (5 mm and 4 mm), *V. parahemolyticus* (4 mm and 5 mm) respectively. On the contrary least inhibition was observed against *S. typhi* (2 mm and 2 mm). The crude mucus was ineffective against the other bacteria and the fungal strains. The comparative antibacterial effect of the mucus from the two marine fish's *C. arel* and *A. caelatus* by using standard drug tetracycline are also shown in the Figure

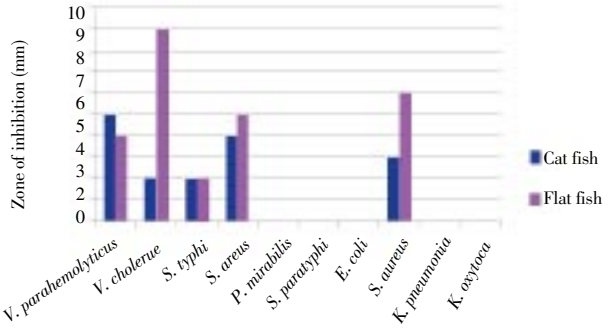

Figure 2. Antibacterial activity of the fish mucus against human pathogens.

3.2. Protein estimation

The amount of protein present in the mucus extract of *C. arel* was 11.6% and *A. caelatus* extract contains 12.3% respectively.

3.3. Haemolytic assay

The crude mucus as well as the fractions produced pronounced hemolytic activity on chicken and goat erythrocytes (Figure 3&4). Hemolytic factors were present in the crude mucus as well as in all the fractions, but differed considerably depending on the type of blood used. Chicken blood, was the most vulnerable to lysis provoked by the *C. arel* mucus. The crude extract of chicken blood showed maximum of 64 HU/mg for *C. arel* and 8 HU/mg for *A. caelatus* and the goat blood showed maximum of 32 HU/mg for *C. arel* and 32 HU/mg for *A. caelatus*.

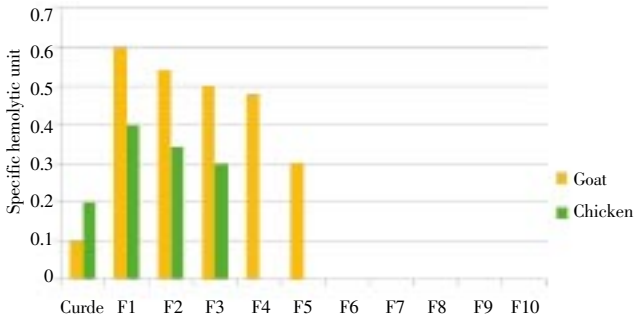

Figure 3. Haemolytic activity of *A. caelatus* mucus against chicken and goat blood.

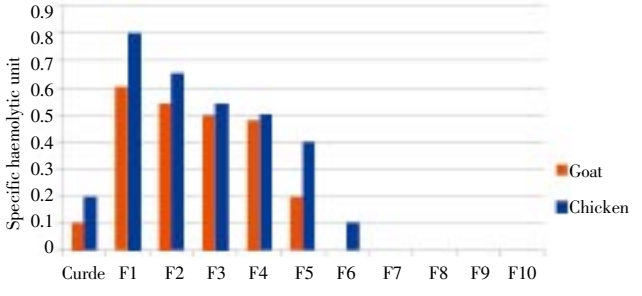

Figure 4. Hemolytic activity of flat fish, *C. arel* epidermal mucus against chicken and goat blood.

3.4. FTIR studies

The FT-IR spectrum of crude mucus, obtained from fish, reveals characteristic functional groups showed in the (Figure 5 & 6). A stretching of C-O-C, C-O at 1 000-1 200 cm^{-1} corresponds to the presence of carbohydrates. Absorption peaks centered on 910-665 and 690-515 cm^{-1} corresponds to N-H was of primary amine and CX stretch of alkyl halides, respectively. IR peak observed in the range of 2 350-2 360 cm^{-1} may be of CO_2 adsorption or asymmetric stretching of group N-C-O. The FT-IR spectrum of mucus from fish confirms the presence of primary amine-group, aromatic-compound, halide-group, aliphatic alkyl-group and polysaccharides (carbohydrates). Consequently, IR spectra may be attributed to the presence of alkyl amine and/or cyclic amine with polysaccharides in the mucus.

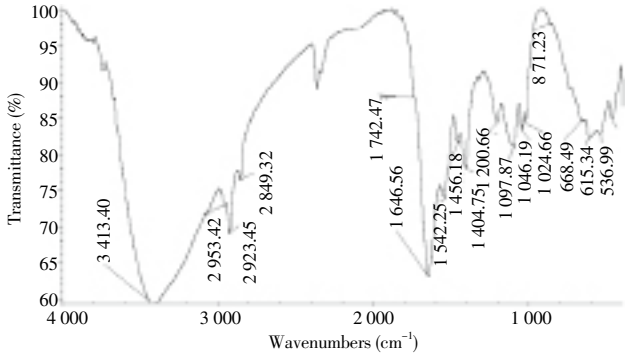

Figure 5. FT-IR analysis of *A. caelatus* fish epidermal mucus.

3.5. Immunomodulatory activity

Preliminary phytochemical investigation reveals that the presence of tannins[14]. The neutrophil locomotion and chemotaxis showed significant activity at all concentrations; fractionated extract have showed significant activity at 50 μg/mL concentrations (Table 1). In case of phagocytosis of killed *C. albicans*, extract showed significant activity even at low concentration of 10 μg/mL concentrations (Table 1-3). *In vitro* Tetrazolium qualitative tests, extract showed significant activity (Figure 7). The extract showed predominantly very good at 50 μg/mL concentrations.

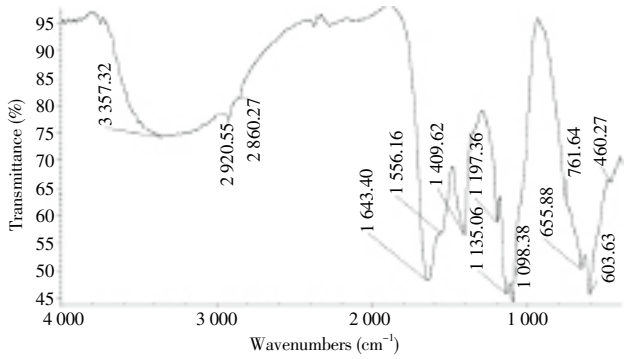

Figure 6. FT-IR analysis of *C. arel* fish epidermal mucus.

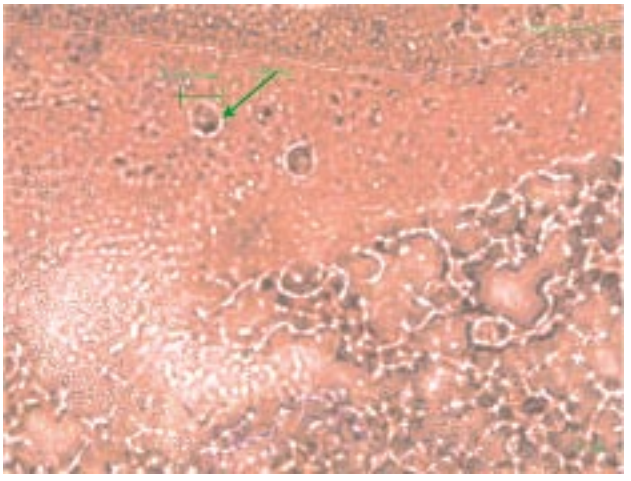

Figure 7. Immunomodulatory activity of fish mucus against blood neutrophils.

Table 1
Neutrophil locomotion and chemotaxis activity of fish mucus.

Mucus extracts	Concentration				Control PBS	Casein
	10 μg/mL	20 μg/mL	50 μg/mL	100 μg/mL		
A. caelatus	100	127	128	120	15	160
C. arel	97	125	130	119	14	160

Table 2
Phagocytosis of fish mucus against *C. albicans*.

Mucus extracts	Concentration				Control(MPN)
	10 μg/mL(MPN)	20 μg/mL(MPN)	50 μg/mL(MPN)	100 μg/mL(MPN)	
A. caelatus	4	4	5	5	4-5
C. arel	5	4	4	5	4-5

Very good activity at 100 μg/mL. MPN= Mean particle number of *C. albicans*.

Table 3
Qualitative nitroblue tetrazolium test (NBT)(%).

Mucus extracts	Concentration				Control	Endotoxin
	10 μg/mL	20 μg/mL	50 μg/mL	100 μg/mL		
A. caelatus	48	50	52	45	22	45
C. arel	48	49	51	47	22	47

Very good activity at 10 μg/mL.

4. Discussion

Many organisms possess antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to compete with conventional antimicrobials obtained from microorganisms[1,6,15]. Fish mucus is multifunctional material, which plays a major role in communication, resistance to disease, respiration, ionic and osmotic regulation, feeding, nest building, reproduction and excretion[16]. In the present study the crude mucus of the two marine fishes shows the antimicrobial activity against the pathogens. As similar result was observed in *Channa punctatus* and *Cirrhinus mrigala*[6]. Antimicrobial compounds have been found associated with and dispersed from the epithelial mucus-secreting cells of fishes[17]. Inhibition effect may be due to the pore forming properties against several bacterial strains and these suggested that fish secrete antibacterial proteins able to permeablize the membrane of the target cell and thus act as a defense barrier. The antibacterial activity may be due to the antibacterial glycoproteins present in the mucus able to kill bacteria by forming large pores in the target membrane[18-22]. The results of the present study support the folkloric usage of fishes and suggest that the mucus of fishes possess certain constituents with antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. The FT-IR analysis of the mucus of the two marine fishes shows distinct spectral profile which confirms the presence of primary amine-group, aromatic-compound, halide-group, aliphatic alkyl-group and polysaccharides (carbohydrates). The results of the *in vitro* PMN function test showed a significant increase in the percentage phagocytosis and phagocytic index for successive crude and fractionated extracts. This indicates that these extracts enhance the phagocytic efficacy of the PMN cells by causing more engulfment of the *Candida* cells versus control, thereby stimulating a non-specific immune response. The final step of phagocytosis is the intracellular killing of microorganisms by the neutrophils, which is dependent on metabolic thrust generated through the hexose monophosphate shunt activation, and activation which also necessary for the normal microbicidal activity[23,24]. The extract of *A. caelatus* and *C. arel* was found to have a significant immunostimulant activity. These results are encouraging enough to pursue structure elucidation of the active components. The present study also showed that the mucus of *C. arel* and *A. caelatus* could be a potential source of novel antimicrobial components for human health related applications. Further studies on the characterization of the antimicrobial substances in these acidic mucus extracts will further our understanding of the composition and function of the antimicrobial components in the mucosal defense mechanism of these fish species.

Conflict of interest statement

We declare that we have no conflict of interest.

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