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Biochemical characterization, antimicrobial and hemolytic studies on skin mucus of fresh water spiny eel *Mastacembelus armatus*

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ABSTRACT

Objective: To characterize the biochemical, antimicrobial and hemolytic activities of *Mastacembalus armatus* skin mucus. **Methods:** Antimicrobial and antifungal activities of mucus extractions against human and fish pathogens were tested along with ampicillin as control. Hemolytic activity of the extraction was evaluated against sheep and cow blood cells. Amino acid and fatty acid profiles were analyzed by HPLC and gas chromatography in the mucus of fish. SDS-PAGE analysis of mucus and muscle tissue was done. Oneway-ANOVA was performed against all extraction and pathogens, amino acids and fatty acids. **Result:** All the mucus extracts exhibited higher inhibitory activity than antibiotic ampicillin against bacterial and fungal pathogens. The hemolytic activity was increased with higher mucus concentrations in both sheep and cow blood cells. The protein content soluble and insoluble fractions of mucus were 63.22 μg/g and 55.79 μg/g, respectively. Out of 17 amino acids leucine was higher (8.54 mole %) in soluble gel, and glutamic acid was higher (6.92 mole %) in the insoluble gel, Histidine was very low (*i.e.* 0.20 mole %) both in soluble and (0.30 mole %) insoluble gel. In SDS-PAGE analysis, 6 bands of mucus and 9 bands of muscle were observed. **Conclusions:** The soluble and insoluble proteins are responsible for antimicrobial and hemolytic activity, these results indicate that mucus gel was prospective applications in fish and human therapeutics.

1. Introduction

Mastacembelus armatus (*M. armatus*) is the largest spiny eel and an economically important food fish of Asian countries. It is a medicinally important fish[1] and its epithelial surface secretes large amount mucus (0.5% to 1.0% of body weight) compared with other teleost. The mucus protects the skin from pathogens and suspended particles and its alarm substance mucin has potential of antimicrobial and noxious properties[2]. Mucus plays an important role in the prevention of colonization of parasites, bacteria and fungi[3]. The functional properties of the mucus depend on its capacity to form a gel on the epithelial surface[4]. This mucus is secreted by the epidermal goblet cells composed mainly of water and gel forming macromolecules such as mucins, and other glycoproteins, *etc*[5].

A key innate immune component is the mucus layer on the surface of fish, which is secreted by goblet or mucus cells in the epidermis and functions as a physical and biochemical barrier between fish and its aquatic environment. It also contains a variety of biologically active substances that function as innate immune factors[6]. The mucus layer on the surface of the fish is continuously replaced which possibly prevents stable colonization by parasites, bacteria and fungi. Skin secretions contain a wide variety of polypeptides with antimicrobial properties. Proteases are considered to be antimicrobial proteins which involved in the regulatory production of antimicrobial peptides. In addition fish mucus also contains a variety of biologically active substances such as lysozyme, lectins, flavoenzymes, immunoglobulins,

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C-reactive protein, apolipoprotein A-I and antimicrobial peptides which gives protection to fish from potential pathogens[7]. Mucus is the slimy secretion consisting of mucins and combination of other substances such as inorganic salts, immunoglobulins and lipids suspended in water giving it characteristic lubricating properties[8]. Antimicrobial peptides are important in the first line of the host defense system of many animal species. Antimicrobial peptides act as therapeutic agents[9]. Antimicrobial peptides of fish epidermal mucus has shown a broad spectrum of activity that is 12-100 times more potent than of amphibian AMPs against various fish and human pathogens .Their value in innate immunity lies in their ability to function without either specificity or memory.

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. Both the soluble fraction and insoluble fraction of the proteinaceous gel showed strong hemolytic activity[10]. The present investigations was carried out to determine antibacterial and antifungal activity and also to study the amino acid and fatty acid profiles in the mucus of *M. armatus*.

2. Materials and methods

Spiny eel *M. armatus* was collected from Kaveripatti (Latitude 11° 32′ 26″ N Longitude 77° 43′ 50″E) and Kotthampatty (Latitude 11° 31′ 34″ N Longitude 77° 43′ 8″ E) of Cauvery river, Erode, Tamil Nadu, India from June to November of 2010. The fishes were acclimatized to artificial cave fitted tanks. Mucus was collected from the fresh fish by carefully scraping from the surface of the body using spatula and stored in sterile containers (Plate-I). The mucus was centrifuged at 15 000 rpm for 15 minutes and the supernatant was collected and to lyophilized in DRDO Centre for Life Sciences, Bharathiar University, Coimbatore, India. The lyophilized powder sample was stored at -4℃ for further analysis.

2.1. Extraction of mucus

Extract-A: Aqueous extract was prepared by 1 mg of lyophilized mucus sample mixed in 1 mL of phosphate buffer solution. Extract- B: 1 mg of lyophilized mucus sample was boiled in 1 mL of 10% acetic acid for 5 minutes, cooled, then centrifuged at 18 000 rpm for 35 min at 4℃. The supernatant was evaporated overnight and dissolved in distilled water. Extract-C: The lyophilized mucus sample 1 mg/mL was suspended in 95% of ethanol and centrifuged at 10 000 rpm for 30 min. The supernatant was allowed to evaporate and redissolved in 95 % ethanol for 3 times. The ethanol extract was pooled evaporated and suspended in distilled water to get a final volume of 50 mL and extracted with dichloromethane (CH_2Cl_2). Extract-D: The lyophilized extract of C was dissolved in distilled water and 5% dimethylsulfoxide.

2.2. UV Spectral analysis of mucus extraction

UV absorbance curve of each extract dilutions were determined by JASCO UV 2D Spectrophotometer, Japan. The UV absorbance curve was recorded by scanning the wave length between 200 to 1 000 nm. Maximum absorbance and peak reading were plotted using ORIGIN 6.0 software.

2.3. Antimicrobial activity

2.3.1. Antibacterial activity

Antibacterial activity of mucus extractions-A, B, C and D were determined against 5 bacterial strains of Human pathogens *viz. Escherichia coli, Vibrio cholera, Staphylococcus aureus, Salmonella typhi, Klebsiella pneumonia* and 5 of fish pathogens *i.e., Yersinia ruckeri, Aeromonas hydrophila, Aeromonas formican, Aeromonas liquefaciens* and *Pseudomonas aeruginosa* (IMTECH, Chandigarh, India). Antimicrobial activity was measured using the standard diffusion disc plate assay[11]. Antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 3 mm diameter well and measured in millimeter.

2.3.2. Antifungal activity

Anti fungal activity of mucus extraction was determined against 6 fungal strains *viz. Aspergillus niger, Aspergillus flavus, Candida albicans, Cryptococcus neoformans* and *Mucor* sp. The fungal strains were prepared from the colonies of 24 hours culture on potato dextrose agar medium and adjust with McFarland density to obtain final concentration of approximately 104 CFU/mL. 5% of diluted extracts (A, B, C and D) was carefully sprayed on each fungal strain medium, and incubated at 28℃. Inhibition zones were measured after 24 hours of incubation. Standard antibiotic was used as antifungal control.

2.4. Preparation of erythrocytes suspension

The crude extract of *M. armatus* mucus was assayed by goat and cow blood followed by the method of Paniprasad and Venkateshwaran[12]. An anticoagulant 5% of EDTA solution was added to the blood samples. The blood samples were centrifuged thrice at 5 000 rpm for 5 minutes at along with saline phosphate buffer (pH 7.4). The supernatant was discarded and about 1.0 mL of packed RBC was resuspended in saline phosphate buffer to obtain a 1% RBC suspension used for analysis.

2.5. Amino acid and fatty acid analysis

The amino acid composition of both soluable and insoluable gels were estimated following the method of Yamamoto *et al*[13]. Fatty acid profile of mucus extract was estimated as per the method of Mat Jais *et al*[14].

2.6. SDS-PAGE

SDS-PAGE was performed in 12% separating gels (*M. armatus* tissue and mucus gel protein) to estimate the molecular weight of the active components of the proteinaceous gel using the standard method [15] and the bands were visualized using silver staining[16].

2.7. Statistical analysis

One way ANOVA was performed on all data to compare treatment effects, means were separated using Duncan's Multiple Range Test (SPSS 13Version).

3. Results

3.1. Spectral property

The lyophilized mucus extractions of *M. armatus* were subjected to UV spectral analysis (100 to 1 000 nm). In all the extractions major of peaks were observed between 200 nm to 400 nm. The sharp peaks (200-400 nm) were observed in organic solvents extracts (C and D) than A and B (Figure 1. A, B, C and D).

3.2. Antimicrobial activity

Fish mucus extractions (A-D) showed a higher level of restriction zone against human bacterial strains when compared to antibiotics-ampicillin (Table 1). It was statistically significant for both treatment and bacterial strains at 0.01% level and between treatment and bacterial strains it also showed significance at 0.05% level. *M. armatus* mucus extraction showed an excellent inhibition zone against

Table 1
Antibacterial activity *M. armatus* mucus against human pathogens (mean±SD).

Dilution (1 mg/mL)	*Escherichia coli*	*Vibrio cholerea*	*Staphylococcus aureus*	*Salmonella paratyphi*	*Klebsiella pneumonia*
Extract A	12.00±2.00^{a}	9.00±3.00^{a}	6.00±1.00^{bc}	10.00±3.00^{a}	7.00±1.00^{a}
Extract B	14.00±1.00^{a}	7.00±2.00^{ab}	9.00±4.00^{ab}	11.00±3.00^{a}	9.00±1.50^{a}
Extract C	11.00±1.00^{a}	6.00±1.00^{ab}	6.00±1.00^{bc}	11.00±1.00^{a}	7.00±1.00^{a}
Extract D	13.00±3.00^{a}	10.00±3.00^{a}	11.00±1.00^{a}	9.00±1.00^{a}	8.00±4.00^{a}
Amphicilin	4.00±3.00^{b}	3.00±1.00^{b}	4.00±1.00^{c}	7.00±1.00^{a}	6.00±1.00^{a}
Treatment			15.99**		
Bacteria			8.83**		
Treatment× Bacteria			2.19*		

**Significant at 0.01 level; *Significant at 0.05 level, ns- Not significant. Mean in a column followed by a different letters are significantly(P<0.05).

Table 2
Anti bacterial activity of *M. armatus* mucus against fish pathogen (mean±SD).

Dilution (1 mg/mL)	*Yersinia ruckeri*	*Aeromonas hydrophila*	*Pseudomonas aeruginosa*	*Aeromonas formican*	*Aeromonas liquefaciens*
Extract A	12.00±1.00^{ab}	16.00±2.00 ^{a}	11.00±3.00^{a}	15.00±3.00^{a}	15.0±1.00^{a}
Extract B	12.00±1.50^{ab}	15.00±2.00^{a}	12.00±1.00^{a}	13.00±1.00^{a}	13.0±3.00^{ab}
Extract C	13.00±2.00^{ab}	14.00±1.00^{a}	11.00±1.00^{a}	15.00±1.00^{a}	13.00±1.00^{ab}
Extract D	15.00±3.00^{a}	13.00±1.00^{a}	10.00±3.00^{a}	16.00±1.00^{a}	12.00±1.00^{ab}
Ampicillin	11.00±2.00^{b}	9.00±1.00^{b}	8.00±3.50^{a}	8.00±1.00^{b}	9.00±2.50^{b}
Treatment			12.48**		
Bacteria			5.04**		
Treatment×Bacteria			1.32^{ns}		

**Significant at 0.01 level; *Significant at 0.05 level, ns- Not significant. Mean in a column followed by a different letters are significantly(P<0.05).

Table 3
Antifungal activity of *M. armatus* mucus against fungal pathogens (mean±SD).

Dilution(1 mg/mL)	*Aspergillus niger*	*Aspergillus flavus*	*Candida albicans*	*Cryptococcus neoformans*	*Mucor* sp.
Extract A	7.30±1.52^{b}	8.00±1.20^{a}	8.00±3.00^{ab}	9.00±2.00^{a}	10.00±1.00^{a}
Extract B	6.60±1.00^{b}	6.00±1.00^{b}	9.00±1.00^{a}	8.00±1.00^{ab}	9.00±1.00^{a}
Extract C	7.00±1.32^{b}	8.00±1.00^{a}	7.00±1.00^{ab}	8.00±3.00^{ab}	9.33±2.53^{a}
Extract D	10.30±0.55^{a}	9.00±1.00^{a}	7.00±3.00^{ab}	7.00±0.50^{ab}	8.00±1.50^{a}
Ampicillin	3.00±1.35^{c}	4.00±1.00c	4.00±1.00^{b}	5.00±1.00^{b}	4.00±1.00^{b}
Treatment			20.71**		
Fungi			5.04**		
Treatment× Fungi			1.66^{ns}		

**Significant at 0.01 level; *Significant at 0.05 level, ns- Not significant. Mean in a column followed by a different letters are significantly(P<0.05).

Table 4.
Hemolytic activity of *M. armatus* mucus against sheep and cow blood cells (Mean±SD).

Con. of mucus(μg/mL)	Hemolysis of sheep blood (%)				Hemolysis of cow blood (%)			
	Ex A	Ex B	Ex C	Ex D	Ex A	Ex B	Ex C	Ex D
10	0.00±0.00^{f}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00d	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{c}
20	0.08±0.42^{ef}	0.03±0.01^{cd}	0.04±0.00^{cd}	0.07±0.03^{cd}	0.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{c}
30	0.12±0.04^{d}	0.05±0.02^{cd}	0.07±0.01^{bcd}	0.09±0.028^{bcd}	0.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{c}
40	0.13±0.02^{cde}	0.06±0.02^{bcd}	0.09±0.02^{bcd}	0.11±0.01^{bc}	0.01±0.0^{bc}	0.02±0.01^{a}	0.01±0.01^{b}	0.02±0.01^{c}
50	0.16±0.03^{cde}	0.08±0.01^{bcd}	0.11±0.01^{abc}	0.13±0.01^{bc}	0.02±0.01^{bc}	0.03±0.04^{a}	0.02±0.01^{ab}	0.04±0.01^{bc}
60	0.18±0.01^{b-e}	0.10±0.02^{a-d}	0.12±0.04^{abc}	0.14±0.02^{bc}	0.05±0.01^{abc}	0.04±0.03^{a}	0.04±0.00^{ab}	0.06±0.01^{bc}
70	0.20±0.04^{bcd}	0.12±0.03^{abc}	0.14±0.01^{abc}	0.15±0.01^{bc}	0.06±0.01^{abc}	0.04±0.03^{a}	0.05±0.02^{ab}	0.08±0.0^{bc}
80	0.23±0.03^{ab}	0.13±0.01^{abc}	0.15±0.02^{ab}	0.17±0.02^{bc}	0.08±0.0^{abc}	0.05±0.04^{a}	0.05±0.01^{ab}	0.09±0.0^{bc}
90	0.27±0.03^{ab}	0.16±0.02^{ab}	0.17±0.04^{ab}	0.21±0.01^{abc}	0.10±0.01^{ab}	0.08±0.01^{a}	0.07±0.01^{ab}	0.11±0.02^{a}
100	0.32±0.03^{a}	0.19±0.01^{a}	0.21±0.01^{a}	0.25±0.02^{a}	0.14±0.01^{a}	0.11±0.01^{a}	0.10±0.00^{a}	0.13±0.02^{b}
Con. sheep blood	24.79**							
Con. cow blood	11.98**							
Blood of both animals	97.56**							
Con. × Ext	1.49^{ns}							
Con. × Blood	2.63**							
Ext. × Blood	3.27*							
Con. × Ext × Blood	1.61*							

Con-Concentration, Ext-Experiment.
**Significant at 0.01 level; *Significant at 0.05 level, ns- Not significant. Mean in a column followed by a different letters are significantly(P<0.05).

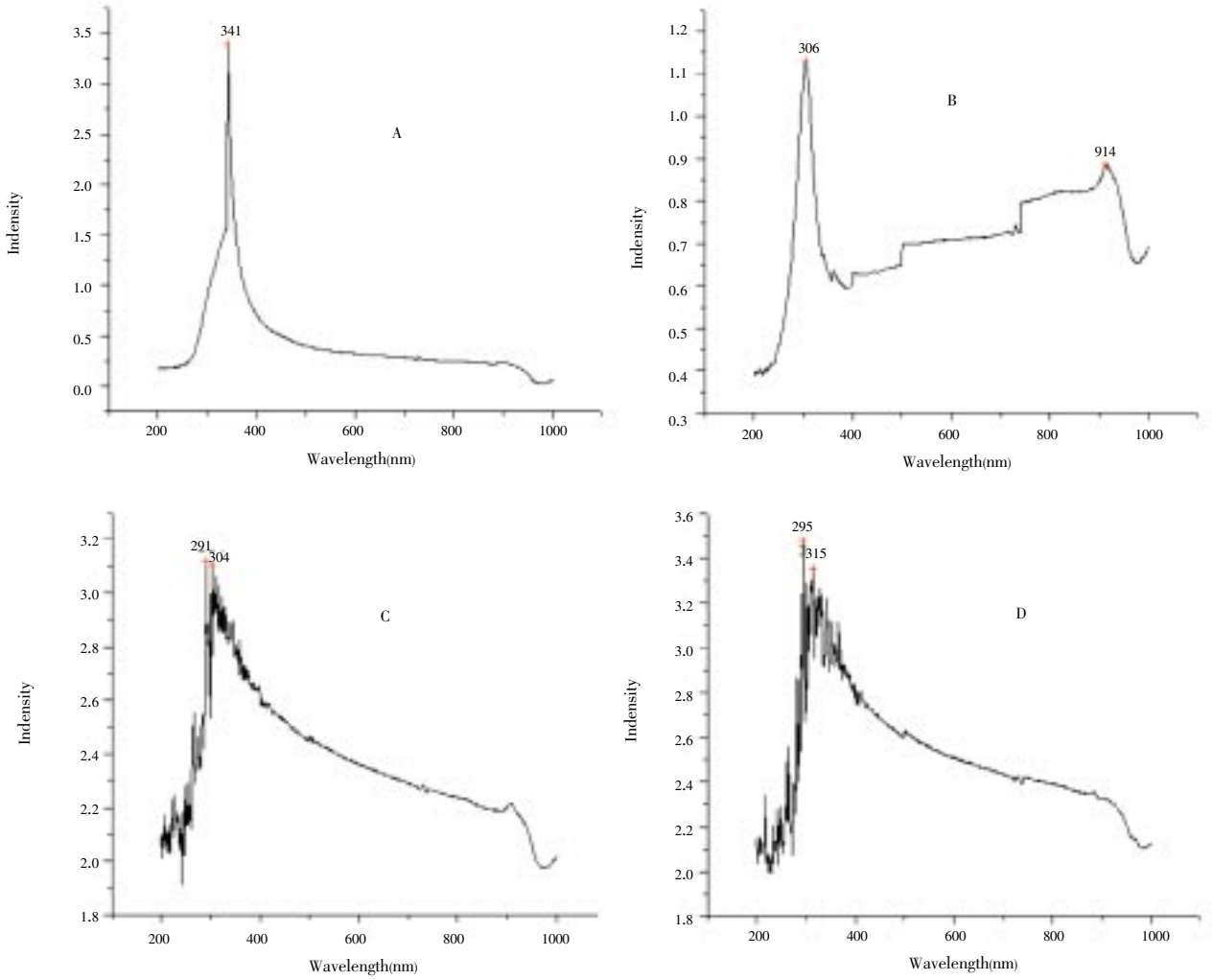

Figure 1. Spectral analysis of *M. armatus* mucus extracts (A, B, C and D).

all fish bacterial pathogens than commercial antibiotic ampicillin (Table 2). The bacteria were significant at 0.01%, but no significance was established between treatment and bacteria. The fish mucus extraction (A-D) showed higher

antifungal activity than control, and higher fungal activities than the antibiotics (Table-3). Statistical significance was observed in both treatment and fungal strains at 0.01% level, but insignificance between treatment and fungal strains.

3.3. Hemolytic activity

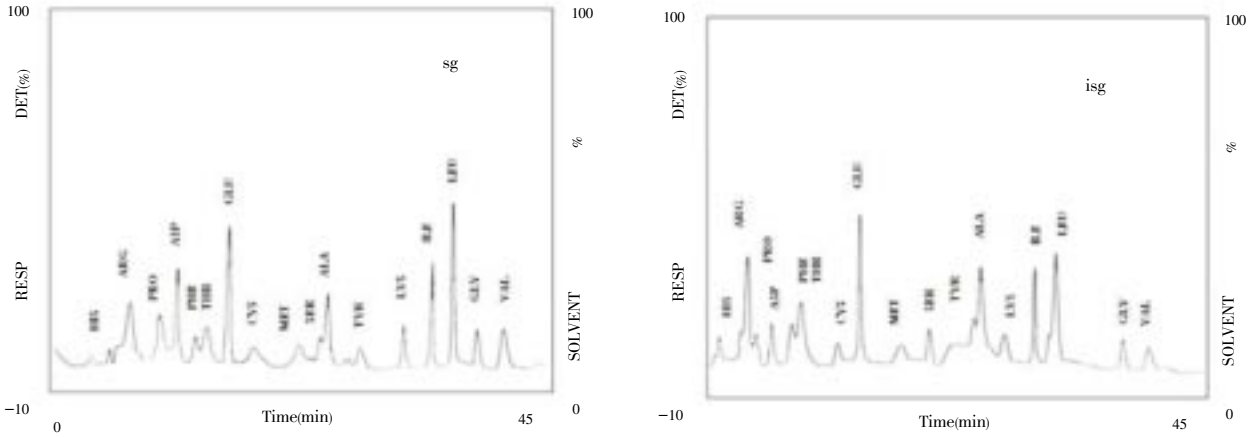

Figure 2. The chromatograms showing amino acid profiles of *M .armatus* mucus sg- soluble portion isg- insoluble portion.

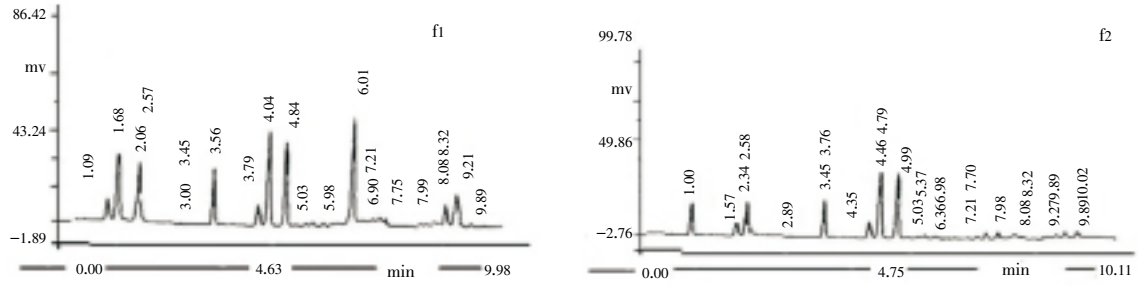

Figure 3. The Gas Chromatogram showing the fatty acid profiles of *M.armatus* f1-soluble gel and f2-insoluble gel.

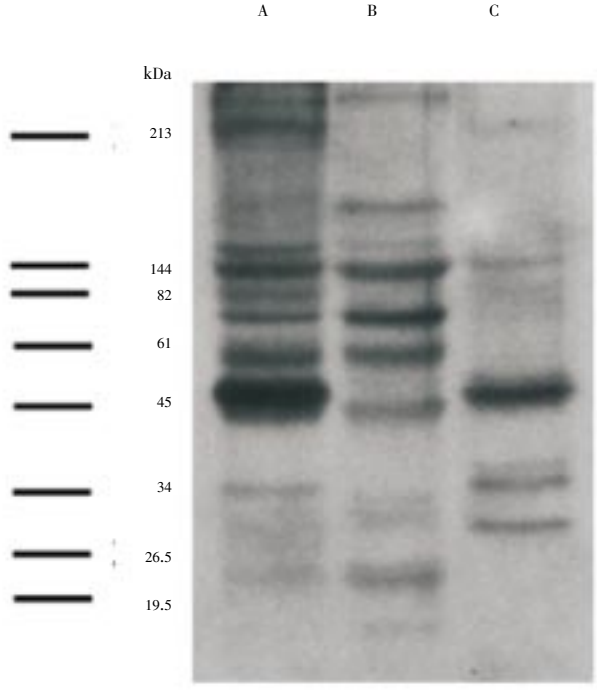

Figure 4. SDS-PAGE of proteinaceous gel secretion of *M. armatus* mucus and muscle protein.

In the present study the hemolytic activity of *M. armatus* mucus gel lyophilized powder extraction (Aqueous and organic solvents) showed strong activity. The extraction A showed high lysis activity in cow and sheep blood. The property of all other extractions were higher than A. The activity was decreased with dilution of extracts. The statistical significance was observed in mucus concentration × sheep blood, mucus concentration × cow blood, at 0.01% level. A significant value of 0.05% was recorded between extraction and blood samples, mucus concentration × extractions × blood samples.

3.4. Amino acid

The amino acid profile of fish mucus both in soluble and insoluble gel mole percentage were recorded (Figure 2). The amino acids leucine (8.54 %) and glutamic acid (6.87%) were recorded at maximum level, cysteine and histidine (0.20 and 0.40%) were recorded at minimum level in soluble gel, where as in insoluable gel glutamic acid and isoleucine (7.59 and 6.92%) were recorded at maximum and tyrosine (0.72%) and histidine (0.30%) recorded at minimum. Higher level of glutamic acid, leucine and isoleucine in the soluble and insoluble gel mucus involved in antimicrobial activity and thus exhibited physiological barrier for these microbes.

3.5. Fatty acid

The fatty acid profile of *M. armatus* of soluble and

insoluble gels showed saturated and unsaturated fatty acids. The soluble gel fraction represented a total saturated fatty acid content of 9.29% with high behanic acid (3.57%) and low lignoceric acid (0.02%). The total percentage of MUFA of soluble gel was 34.57% with maximum of oleic acid (17.82%) and minimum of palmitolic acid (2.32%),. The PUFA of soluble gel was 65.39% with high linoleic acid (21.65%) and low of stearidonic acid (0.54%). In the insoluble gel the total percentage of saturated fatty acids was 8.24%, with maximum of 2.34% of palmitic acid and absence of lignoceric acid. Similarly, in the same gel fraction unsaturated fatty acids MUFA included (25.49%) high level of oleic acid (13.43%) and low level of palmitolic acid (2.03%) where as PUFA (56.14%) with maximum of linoleic acid (19.14%) and absence of stearidonic acid.

3.6. SDS-PAGE

The molecular weight of proteins in the mucus extractions of *M. armatus* was subject to SDS-PAGE analysis. Standard protein marker was used to determine the molecular weight of muscle and mucus proteins. In both the samples a prominent band was observed at 45kDa, a weak band was observed at 34kDa and 144kDa.

Mean in a column for (Extracts and antibiotics) treatment followed by a same letter's are not significantly ($P<0.05$) different according Duncan's Multiple Range Test.

Mean in a column for (Extracts and antibiotics) treatment followed by a same letter's are not significantly ($P<0.05$) different according Duncan's Multiple Range Test.

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Mean in a column for (Extracts and antibiotics) treatment followed by a same letter's are not significantly ($P<0.05$) different according Duncan's Multiple Range Test.

4. Discussion

The UV spectral analysis of *M. armatus* shows sharp peak values in between 200-400 nm in all the mucus extracts. Jill *et al*[17] reported that most of the fish mucus has more than one peak in spectral analysis. The absorbance of all type of mucus was below 290 nm, due to structural components of mucus, such as nucleic acid and proteins[18], gill secretions[19].

The *M. armatus* mucus organic extracts (C and D) shows high level of inhibition zone against both bacterial and fungal pathogens. Many workers previously demonstrated the antimicrobial property of epidermal mucus in fishes: Common carp - *Cyprinus carpio*[20]; Hag fish- *Myxine glutinosa*[21]; Cat fish-*Arius maculatus*[22]; Eel fish - *Anguilla anguilla*[23]. Videler *et al*[24] stated that mucus secreted by external epithelial globlet cells possess biochemical antibiotics compounds. In Gold fish (*Carassius auratus*) the primary activating antimicrobial compound was a serum protein- transferrin was next to epidermal mucus[25]. Ellis[26] reported that epidermal mucus contains variety of antimicrobial components such as AMPs, lysozomes, proteases and lecithins. Antimicrobial compounds have been found associated with and dispersed from the epithelial mucus secreting cells of fishes[27]. The innate immunity of mucus has broad antimicrobial effect, and have been identified in a variety of multicellular organisms [28].

The mucus secretion of *M. armatus* has proteinaceous substances which show potent bioactivity (hemolytic) when mixed with blood cells of sheep and cow. Mucus extracts such as A and D exhibits high level of hemolytic activity when the concentration increases. Some antimicrobial agents present in the mucus of bony fishes which bind with microbes and destroy the blood cells (Hemolysis)[29]. Hellio *et al*[30] reported that lysozyme in the mucus has bacteriostatic properties and was upiquitous in its distribution among living organisms.

In the silver staining method the bands of *M. armatus* at 45 kDa, 34 kDa and 144 kDa were recorded in both mucus and tissue samples. Many researcher isolated proteins from different tissue and mucus from various fishes - Atlantic hagfish [31]; Winter flounder [32]; Atlantic halibut[33].

The present study indicated that the organic mucus extracts have potential antimicrobial activity and indicated that the soluble and insoluble proteins are responsible for the defensive purpose again the invading bacterial and fungal pathogens. The mucus secretion of the spiny eel *M. armatus* is proteinaceous and shows potential hemolytic activity. Thus the mucus of this fish remains an interesting source for isolating a new antimicrobial component and need further characterization.

Conflict of Interest Statement

We declare that the present work has no conflict of interest.

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