

## Preliminary study on antimicrobial activities of skin mucus from by-catch of *Elasmobranch* species

by

Elif Özlem Arslan Aydoğdu<sup>\*,1</sup>, Mert Kesiktaş<sup>2</sup>, Nazmiye Ozlem Sanli<sup>3</sup>, Nihal Doğruöz Güngör<sup>1</sup>, Serap Sancar<sup>4</sup>, Taner Yıldız<sup>5</sup>, Emre Yemişken<sup>2</sup>

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<sup>1</sup>Istanbul University, Faculty of Science, Department of Biology, Fundamental and Industrial Microbiology Division, 34134 Vezneciler, Istanbul, Türkiye

<sup>2</sup>Istanbul University, Faculty of Science, Department of Biology, Hydrobiology Division, 34134 Vezneciler, Istanbul, Türkiye

<sup>3</sup>Istanbul University, Faculty of Science, Department of Biology, Biotechnology Division, 34134 Vezneciler, Istanbul, Türkiye

<sup>4</sup>Istanbul University, Faculty of Science, Department of Biology, Molecular Biology Division, 34134 Vezneciler, Istanbul, Türkiye

<sup>5</sup>Istanbul University, Faculty of Aquatic Sciences e, Department of Fisheries and Seafood Processing Technology, 34134 Vezneciler, Istanbul, Türkiye

\* Corresponding author: [eoarslan@istanbul.edu.tr](mailto:eoarslan@istanbul.edu.tr)

### Abstract

Skates and rays, which are widely encountered in the by-catch of fisheries activities from the Sea of Marmara and banned for sale by regulation, are species that are discarded if caught. For this reason, in our study, we aimed to determine the bioactive potentials of these species, considered fishing waste, by investigating the skin secretions and microbial flora. In our study, both the skin flora and mucus contents of the discarded species *Dasyatis pastinaca* (Linnaeus, 1758), *Myliobatis aquila* (Linnaeus, 1758), and *Raja clavata* (Linnaeus, 1758) caught in the Sea of Marmara were investigated to determine their potential antimicrobial activities. A total of 164 bacteria were isolated from the epidermal mucus of the three batoid species. Antibacterial activity was observed from three isolated bacteria against *Escherichia coli*, Vancomycin-resistant *Enterococcus faecium*, *E. faecalis*, and *Bacillus subtilis*. Additionally, the highest antibacterial activity was observed for skin mucus of *R. clavata*. Mouse fibroblast cell viability was challenged with mucus secretions. *M. aquila* and *R. clavata* mucus secretions exhibited no observable change after 24 and 48 hours. The assays indicated that both the isolates and the skin mucus have potential antimicrobial activity against opportunistic pathogens.

**Key words:** fish skin mucus, antimicrobial activity, batoid, skate, ray

## 1. Introduction

Epidermal components enlist the first barrier between the organism and the environment. The mucus layer covers the body not only for a mechanical protective function but also serves as osmoregulation, chemical communication, social behavior, and protection from abrasion, toxins, heavy metal toxicity, and pathogens (Coello & Khan 1996, Robinette et al. 1998, Fernandes & Smith 2002, Qin et al. 2002, Ullal et al. 2008, Fuochi et al. 2017). The epidermal barrier is the first step of body defense not only for fish species but also for all multicellular organisms. Both the mucosal secretions of the organisms and the presence of microorganisms sheltering as well as their secretions are the factors that strengthen this barrier (Austin & McIntosh 1988, Fouz et al. 1990, Kalidasan et al. 2014). These mucosal secretions, together with the microbial bioactive compounds acquired from the epidermal flora, play an active role in the destruction of pathogens encountered by the organisms. Therefore, the antibacterial activity of these secretions is more effective on the pathogens present in the animal habitat. For this reason, it is seen that even individuals belonging to the same species have secretions that are effective on different pathogens (Ellis 2001, Ritchie 2006, Chau et al. 2013, Abdelmohsen et al. 2014).

Studies show that antimicrobial peptides in these mucus secretions, from epidermal or microflora origin, are the main defence elements against pathogens (Lauth et al. 2002, Shike et al., 2002, Cole et al. 2008, Masso-Silva & Diamond 2014). Several studies from the Atlantic Ocean have examined bacteria associated with fish species and which were shown to produce antimicrobial activity against human pathogens (Yap 1979, Fouz et al. 1990). Studies on the antimicrobial activity of mucus are not limited to fish species. In a study conducted by Ritchie on the coral species *Acropora palmata* in the Caribbean in 2006, it was proved that 20% of the bacterial population living as symbionts on these creatures had antibacterial effects against the tested pathogens (Ritchie 2006).

In recent years, it is observed that studies on the antimicrobial properties of mucus secretions of elasmobranch species have been increasing. Batoidea is a superorder of cartilaginous fish commonly known as rays and skates. These species are considered important predators within marine ecosystems and generally live in demersal habitats. Their morphological properties as dorso-ventrally flattened bodies support moving on the bottom and hiding in the sand (Cortes 1999, Barria et al. 2015, Navia et al. 2017). Skates and rays are commonly taken especially as by-catch of trawl fisheries.

Because batoids are species that move between different water layers, they encounter a wide range of microbial diversity within the marine habitat. In this way, the flora can show great differences both at the species and individual levels. The diversity of microbiota may cause the antimicrobial activity of this barrier to be effective on other microbial species (Cho et al. 2007, Luer 2014, Ritchie et al. 2017).

In total, 38 batoid species were recorded from the Mediterranean Sea (Melendez et al. 2017). *R. clavata* was a single species that had economic importance among other batoid species in the Turkish fish market. However, the most recent regulation of fisheries activities imposed a ban on sales of *R. clavata* (Anonymous 2020). For this reason, these species caught during fishing activities in the Turkish seas are discarded. In these environments, where species diversity is declining day after day due to environmental factors such as climate change and pollution of aquatic ecosystems, as well as uncontrolled hunting practices, it is extremely important to study alternative ways for these species' evaluation.

Due to the misuse of existing antimicrobial agents, the competition against infections caused by antibiotic-resistant strains grows more difficult day by day. The World Health Organisation predicts that by 2050, human deaths from antibiotic-resistant infections will reach 10 million (O'Neill 2016). One of the most important strategies to prevent this scenario is the discovery of new antimicrobial agents. As with the antimicrobial agents discovered so far, the source for the new agents is the examination of unstudied creatures. With this point of view, research on bioactive substances in not only microscopic creatures but also animal and plant species are rapidly continuing. The Marmara Sea is rich in human pathogen species due to urban wastes being discharged with insufficient treatment. As with many species found in this environment, batoids can produce antimicrobial agents against these pathogens as part of their defence mechanism. The same situation is valid for bacterial species living in the aquatic environment and/or in the skin flora of these animals. Therefore, in our study, we preferred the Marmara Sea Elasmobranchii species, which have a high probability of encountering these active compounds due to the pathogen density. In summary, the aim of this study is to determine the antimicrobial effect of mucus samples and culturable bacteria belonging to the skin flora of the discarded *Dasyatis pastinaca*, *Myliobatis aquila* and *Raja clavata* species caught in the Marmara Sea on selected pathogenic species.



## 2. Materials and methods

### 2.1. Epidermal mucus collection

Fifteen specimens of three different fish species were caught by fishermen from the North Sea of Marmara (Hoskoy Coast). Samplings, after being carefully washed with sterile seawater to remove allochthone bacteria, were made from the discarded dead species, brought to the port by the fishermen. Epidermal mucus was obtained from the pectoral fin surfaces with a sterile scoopula, transferred to sterile culture tubes, and frozen and stored at  $-20^{\circ}\text{C}$ . A swab collection of fresh mucus was used to isolate bacteria from the dorsal surfaces of each individual.

### 2.2. Preparation and extraction of mucus

Mucus extraction was based on Vennila et al. 2011. Mucus samples were treated with distilled water, acetic acid (AA) and trifluoro acetic acid (TFA) and used in the experiments. For distilled water extraction, samples were lyophilized overnight followed by mixing mucus powder with distilled water and sonicating twice for a minute each. For AA extraction, the samples were mixed with 0.05 M acetic acid in the ratio of 3:4 and placed in a boiling water bath for 5 minutes. For TFA extraction, a 200  $\mu\text{g}$  sample was dissolved in 2 ml of 0.1% TFA and kept on ice for 2 hours. For all solvents, after extraction the samples were centrifuged at  $12000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , and the supernatants were lyophilized for 24 hours. Following that, the lyophilized samples were dissolved with distilled water and sterilised by a 0.20  $\mu\text{m}$  pore diameter filter. The extracted skin mucus was added in a concentration range from 50  $\mu\text{g} \mu\text{l}^{-1}$  to 6.5  $\mu\text{g} \mu\text{l}^{-1}$  for antimicrobial assays.

### 2.3. Isolation of Bacteria from Epidermal Mucus

Mucus samples were serially diluted in phosphate buffer saline solution. 100  $\mu\text{l}$  aliquots of each dilution were spread onto Marine Agar (MA, Oxoid), 5% sheep blood tryptic soy agar (Blood-TSA, Oxoid), and Tryptic Soy Agar (TSA, Oxoid). After 24–48 hours of incubation at  $30^{\circ}\text{C}$ , all macroscopically and microscopically different colonies were selected to obtain a pure culture. Pure cultures were stored at  $-86^{\circ}\text{C}$  in a 20% glycerol solution. Isolated bacterial strains' relationships between different feedlots and batoid species were statistically analyzed using the one-way ANOVA test. The SPSS 21 package program was found out experiment results.

### 2.4. Determination of the antibacterial effect of isolated bacterial strains from mucus samples

After subculturing on MA, the antimicrobial activity of isolates was evaluated against indicator microorganisms *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), Methicillin-resistant *Staphylococcus aureus* (ATCC 33591), *Staphylococcus aureus* (ATCC 6538), Vancomycin-resistant *Enterococcus faecalis* (ATCC 51299), and *Candida albicans* (ATCC 10231) by using the agar overlay technique. Following 48 h incubation, isolates were suspended turbidimetrically in marine broth at a concentration of  $10^9$  cfu (colony forming unit)  $\text{ml}^{-1}$  by using a densitometer (Biosan, Latvia). 10  $\mu\text{l}$  of each suspension were inoculated on the MA and incubated at  $30^{\circ}\text{C}$  for 3 days, and then cultures were irritated by UV. At this stage, 10 ml sterile warm fused TSA medium containing  $10^8$  cfu  $\text{ml}^{-1}$  indicator strains was overlaid on the irritated cultures. Afterwards, the Petri dishes were incubated at the appropriate temperature for 24–48 hours, and then the clear inhibition zones around the spots were examined (Çandiroğlu & Dođruöz Güngür 2020).

### 2.5. Sequencing and phylogenetic analysis

DNA isolations of isolates, detected to have antimicrobial activity, were performed with the GeneAll Exgene™ Cell SV DNA isolation kit. The PCR method was applied to determine the *16S rRNA* gene region of the isolates. Primers (1492R 3'-GGT TAC CTT GTT ACG ACT T-5' and 27F 3'-AGA GTT TGA TCM TGG CTC AG-5') were prepared as 20 ng of each primer, 10 ng template DNA, 5  $\mu\text{l}$  Reaction Buffer, 0.25  $\mu\text{l}$  MyTaq DNA polymerase and adjusted to a final volume of 25  $\mu\text{l}$  by adding sterile ultrapure water. Amplification was performed as was carried out in Thermal Cycler (Bio-Rad, USA) and programmed at 60 s initial denaturation at  $95^{\circ}\text{C}$ , 30 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 s, annealing for 15 s at  $53^{\circ}\text{C}$ , and extension for 10 s at  $72^{\circ}\text{C}$ . The BLAST analyses of *16S rRNA* sequences were obtained by using the National Centre for Biotechnology Information (NCBI) database (Kaya et al. 2021).

### 2.6. Antimicrobial activity of mucus

*In vitro* antimicrobial activity of the mucus was determined with the broth microdilution test using 96-well microtiter plates as recommended by CLSI protocol (CLSI 2006). Acid extracts of mucus samples were serially diluted in TSB (Tryptic Soy Broth, Oxoid) and an equivalent volume of microorganism

suspensions ( $10^8$  and  $10^6$  cfu ml<sup>-1</sup> for bacteria and yeast, respectively) were added to each well. 200 mg ml<sup>-1</sup> of SiliQUAT [3-(trimethoxysilyl)-propyl, cocodimethylammonium chloride] solution was used as a positive control. 0.05 M acetic acid solution and sterile TSB were used as a negative control. The microtiter plates were then incubated for 24 to 48 h at 37°C for bacteria and 30°C for *C. albicans*. After the incubation period, serial dilutions were made for each well and inoculated on TSA for colony counts (CLSI 2016, Bal & Şanlı 2020).

To evaluate the antimicrobial activity the following formula was used:

$$\text{Reduction percentage of bacteria} = \frac{(C - A) \times 100}{C}$$

Where C is the number of cultivable microorganisms without mucus treatment, and A is the number of cultivable microorganisms after mucus treatment. Experiments were performed in triplicate.

### 2.7. Cell viability assay

Mouse embryonic fibroblast cell line 3T3 cells were grown in DMEM:F12 (1:1) cell media supplemented with 10% fetal bovine serum and with 100 U ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin at a humidified 37°C incubator providing 5% CO<sub>2</sub>.

3T3 fibroblast cells were trypsinized, counted, and seeded on 96-well plates at 10000 cell density per well. Mucus secretions from different species were diluted in cell culture media at 10-1000 µg ml<sup>-1</sup> concentrations. The cells were incubated with mucus secretions for 24 and 48 h and cell viability was determined with MTT assay. At the end of each experiment, 30 µl MTT solution (5 mg ml<sup>-1</sup>) was added to each well, and after 4 h purple formazan crystals were dissolved in 100 µl DMSO. Optic density was measured at 570 nm test wavelength and 630 nm reference wavelength using ELISA reader (BioTek, CA).

### 2.8. SDS-PAGE analysis

SDS-PAGE was carried out according to Laemmli (1970). The protein contents of isolated skin mucoproteins from *R. clavata*, *D. pastinaca*, and *M. aquila* species were determined using Bradford reagent and 40 µg mucoproteins of each sample were denaturated, loaded and separated on the 12% SDS-PAGE gels. The gel was run on a Bio-Rad vertical electrophoretic apparatus. The separated peptides were stained in 0.1% (v/v) Coomassie blue R-250 (Coomassie brilliant blue G 0.025% and acetic acid

10% and destained in 40% methanol and 10% acetic acid for 30 min. The gels were stored in 7% acetic acid solution until they were scanned.

## 3. Results

In the study, when the antimicrobial activity of distilled water, AA and TFA extracts of mucus samples belonging to three species were investigated by the microdilution method, it was determined that only acetic acid extract was effective on the pathogens examined. A total of 164 bacteria were isolated from *M. aquila*, *R. clavata* and *D. pastinaca* epidermal mucus. It was determined that there was a significant relationship between bacterial diversity and fish species ( $p < 0.05$ ,  $p = 0.001$ ). No significant relationship was calculated in the statistical analysis between different feedlots and isolated bacterial strains ( $p > 0.05$ ;  $p = 0.1185$ ). Only 3 isolated bacteria were detected as antimicrobial activity against *E. coli*, VRE, *E. faecalis*, *B. subtilis* (Table 1). Both *Enterococcus faecium* (OM967454) and *Brevundimonas* sp. (OM967455) isolated from *Myliobatis aquila* showed antibacterial activity against *E. coli*. Besides, the *Enterococcus faecium* (OM967454) was observed with antimicrobial effects against VRE, and *E. faecalis*. In addition, *Staphylococcus simulans* (OM967456) isolated from *Raja clavata* was found to have antibacterial effects on *B. subtilis*.

**Table 1**

Isolates from epidermal mucus of fish species and their rates of antimicrobial activity

Species	Number of colony (cfu ml <sup>-1</sup> )	Number of isolates	Antimicrobial activity rates of isolates (%)
<i>Dasyatis pastinaca</i>	2442.5 ± 67.4	64	0
<i>Myliobatis aquila</i>	3665 ± 123.2	78	2
<i>Raja clavata</i>	37.5 ± 1.8	22	5

### 3.1. Mucus extract assays

When the antimicrobial activity of distilled water, AA and TFA extracts of mucus samples belonging to three species were investigated by the microdilution method, it was determined that only acetic acid extract was effective on the pathogens examined. The *in vitro* antimicrobial activity of AA extraction of skin mucus of fishes against selective pathogens is shown in Tables 2 to 4. The results indicated that the highest reduction was examined for mucus from *R. clavata* to bacterial strains. On the other hand, mucus of the



Table 2

Growth inhibition rates (%) of tested strains by the acidic mucus extract of *R. clavata*

Test microorganisms	The concentration of mucus extracts ( $\mu\text{g } \mu\text{l}^{-1}$ )				Negative Control (cfu ml <sup>-1</sup> )
	50	25	12.5	6.25	
<i>E. coli</i> ATCC 25922	> 99.999	> 99.699	> 99.999	> 99.999	$2.23 \times 10^{12}$
<i>S. aureus</i> ATCC 6538			> 99.999	> 99.999	$2.57 \times 10^{11}$
<i>P. aeruginosa</i> ATCC 9027			95.100	94.869	$2.98 \times 10^{13}$
MRSA ATCC 33591			41.539	29.039	$1.24 \times 10^{11}$
VRE ATCC 51299	99.469	98.799	63.179	32.279	$1.89 \times 10^{12}$
<i>C. albicans</i> ATCC 10231	> 99.999	> 99.999	> 99.999	> 99.999	$6.90 \times 10^{11}$

Table 3

Growth inhibition rates (%) of tested strains by the acidic mucus extract of *D. pastinaca*

Test Microorganisms	The concentration of mucus extracts ( $\mu\text{g } \mu\text{l}^{-1}$ )				Negative Control (cfu ml <sup>-1</sup> )
	50	25	12.5	6.25	
<i>E. coli</i> ATCC 25922	73.729	60.169	43.019	7.609	$7.23 \times 10^{13}$
<i>S. aureus</i> ATCC 6538	99.149	85.409	60.579	52.159	$2.09 \times 10^{13}$
<i>P. aeruginosa</i> ATCC 9027	> 99.859	> 92.479	43.699	37.399	$2.38 \times 10^{14}$
MRSA ATCC 33591	> 99.939	> 99.609	99.229	98.089	$3.87 \times 10^{14}$
VRE ATCC 51299	91.249	75.469	24.499	NR	$2.49 \times 10^{10}$
<i>C. albicans</i> ATCC 10231	> 99.999	> 99.999	> 99.999	> 99.969	$8.35 \times 10^{11}$

\* NR: No reduction

Table 4

Growth inhibition rates (%) of tested strains by the acidic mucus extract of *M. aquila*

Test Microorganisms	The concentration of mucus extracts ( $\mu\text{g } \mu\text{l}^{-1}$ )				Negative Control (cfu ml <sup>-1</sup> )
	50	25	12.5	6.25	
<i>E. coli</i> ATCC 25922	99.939	99.909	99.859	78.569	$7.23 \times 10^{13}$
<i>S. aureus</i> ATCC 6538	99.779	97.629	84.499	78.809	$2.09 \times 10^{13}$
<i>P. aeruginosa</i> ATCC 9027	> 97.599	> 94.669	90.889	89.079	$2.38 \times 10^{14}$
MRSA ATCC 33591	> 96.539	> 92.799	89.769	87.189	$3.87 \times 10^{14}$
VRE ATCC 51299	99.959	98.579	NR	NR	$2.49 \times 10^{10}$
<i>C. albicans</i> ATCC 10231	> 99.999	> 99.999	> 99.999	> 99.989	$8.35 \times 10^{11}$

other two species showed a higher inhibition effect to fungal strain. The bacterial reduction in the mucus of *R. clavata* and *M. aquila* species did not decrease in a concentration of  $25 \mu\text{g } \mu\text{l}^{-1}$  mucus. Maximum antibacterial activity for a concentration of  $5 \mu\text{g } \mu\text{l}^{-1}$  was observed against *P. aeruginosa*, *S. aureus* and VRE, respectively in *R. clavata*, *D. pastinaca* and *M. aquila*.

All diluted doses of *R. clavata* mucus solution demonstrated an antimicrobial effect on *E. coli*. Indeed, the minimum antibacterial effect was observed for MRSA and VRE in a concentration of  $12.5 \mu\text{g } \mu\text{l}^{-1}$  and  $6.25 \mu\text{g } \mu\text{l}^{-1}$  of mucus from *R. clavata*.

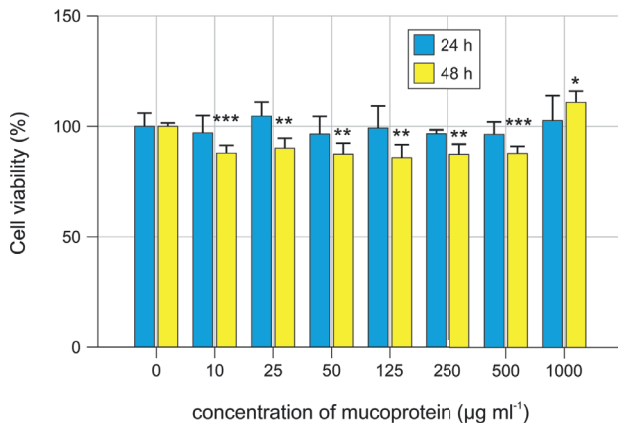
The concentration of  $50 \mu\text{g } \mu\text{l}^{-1}$  of *D. pastinaca* mucus extract exhibited strong antibacterial activity against all bacteria except *E. coli*. Moreover, a 99.999% reduction in the bacterial count was observed in all dilution doses of three different mucus against *C. albicans*.

### 3.2. The effects of different mucus on 3T3 mouse fibroblast cell viability

The effects of the *D. pastinaca*, *M. aquila* and *R. clavata* mucus secretions were tested on mouse fibroblast cells. 3T3 cells were treated with 10-1000  $\mu\text{g } \mu\text{l}^{-1}$  mucus concentrations for 24 and 48 h treatment. We did not observe any changes after 24 h with *D. pastinaca* mucus. On the other hand, the treatment for 48 h to a low decrease in cell viability at 10–500  $\mu\text{g } \mu\text{l}^{-1}$  and only 1000  $\mu\text{g } \mu\text{l}^{-1}$  concentration induced cell proliferation (Fig. 1).

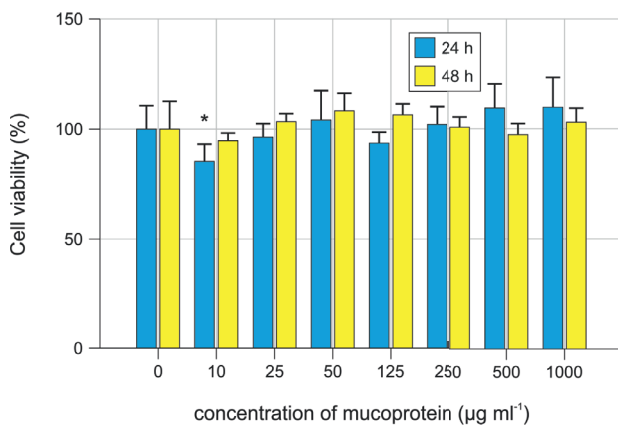
Treatment with the different mucus concentrations of *M. aquila* mucus did not change cell viability significantly in comparison to untreated cells; except only 10  $\mu\text{g } \mu\text{l}^{-1}$  concentration for 24 h treatment led to a low decrease in cell viability (Fig. 2).

The high concentrations of *R. clavata* mucus had no



**Figure 1**

The effects of *D. pastinaca* mucus on cell viability of 3T3 fibroblast cells. Each bar represents the mean  $\pm$  SEM of three independent experiments. The \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control cells.

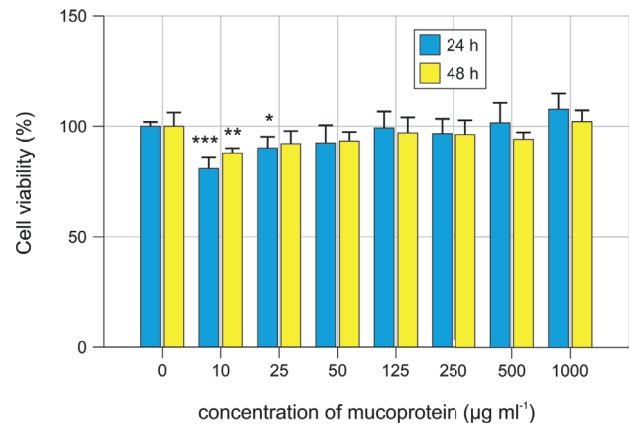


**Figure 2**

The effects of *M. aquila* mucus on cell viability of 3T3 fibroblast cells. Each bar represents the mean  $\pm$  SEM of three independent experiments. The \* $p < 0.05$  versus control cells.

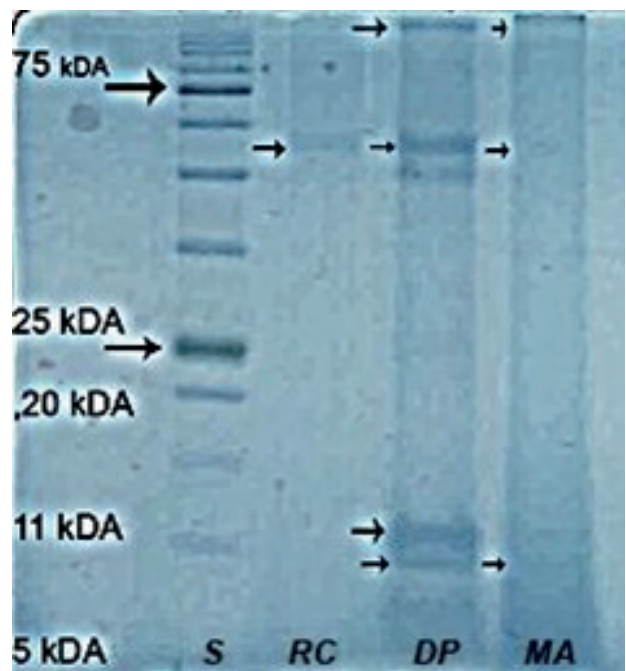
significant effect on the cell viability of 3T3 cells for 24 and 48 h. 10  $\mu\text{g ml}^{-1}$  concentration treatment for 24 and 48 h decreased cell viability to a low level. Similarly, for 24 h treatment with 50  $\mu\text{g ml}^{-1}$  *R. clavata* mucus led to a small reduction in cell viability (Fig.3).

SDS-PAGE results showed. three major subunit bands (~100 kDa, ~50 kDa, and ~11 kDa) and a very faint band between ~11-5kD) resulted from analysis (Fig. 4). *D. pastinaca* and *M. aquila* have subunit bands similar to 11 kDa. *R. clavata* has been represented by 50kDa subunit bands.



**Figure 3**

The effects of *R. clavata* mucus on cell viability of 3T3 fibroblast cells. Each bar represents the mean  $\pm$  SEM of three independent experiments. The \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control cells.



**Figure 4**

SDS-PAGE of mucus and skin extracts of *R. clavata*, *D. pastinaca*, and *M. aquila*. 40  $\mu\text{g}$  mucoproteins of each sample were denatured, loaded and separated on the 12% SDS-PAGE gels under 120 volts. The gels were fixed and then stained in 0.1% (v/v) Coomassie blue R-250. The weight of each protein band was calculated according to the weight of known proteins in the standard (S) loaded into the first well.



## 4. Discussion

Skates and rays have adaptations for living in the demersal zones. The first barrier between fish and the environment consists of skin mucus and the mucus layer includes different biochemical components secreted by epidermal cells. It serves as a mechanical and biological protection for fish.

Indicator bacteria for water pollution are frequently examined in the microbiological studies of the Turkish seas. However, there are insufficient studies in terms of the potential bioactive compounds that may have the bacteria that colonize the surface of organisms such as fish. (Altug et al. 2012, Cardak & Altug 2014, Kalkan & Altug 2015, Ciftci Türetken and Altug 2016). It is important to increase these studies to better understand the defense mechanisms of these creatures living in these seas, which are of high microbial diversity due to different chemical, physical, geological, and climatic effects, and to enable the obtaining of new bioactive compounds.

Several studies from Atlantic Oceans and the Mediterranean Sea have focused on symbiotic bacterial flora in the skin mucus layer and mucus biochemical properties. Two batoid species, *Dasyatis sabina* and *Rhinoptera bonasus*, were found to contain proteins in an aqueous supernatant, and a viscous pellet and mucus pellets were also found to contain symbiotic bacteria with demonstrated antibiotic activity (Luer 2014). 576 strains were isolated from *R. bonasus* and 96 strains were from *D. sabina*. Moreover, of the 1860 bacteria isolated from the epidermal mucus of three stingray species by Ritchie et al. (2017), 311 exhibited antibacterial activity. Despite the aforementioned two studies, in which 17% of the isolates exhibited antibacterial activity, only 2% of the strains obtained in our study showed antibacterial activities against *E. coli*, VRE, *E. faecalis* and *B. subtilis*. The possible reason for this low rate may be the individual limits of the mucus samples. Sampling was limited not only at the species level but also in terms of the number of animals. In other words, today, when the importance of species diversity and number is accepted, the number of samplings has remained at a limited level, as samples are taken from discarded individuals obtained during the hunting of commercially valuable species instead of hunting these animals for the purpose of collecting samples. However, it is of great importance to isolate a bacterium that produces effective antimicrobial agents, especially on resistant strains such as VRE.

According to the literature, secreted material from fish skin has an antimicrobial effect against Gram – and Gram + bacterial strains (Monteiro-Dos-Santos

et al. 2011, Kalidasan et al. 2014, Fuochi et al. 2017). Antimicrobial peptides were exhibited in different fish skin mucus. The inhibition effect against bacterial strains and anti-tumoral traits were demonstrated in the intestinal bioactive compound of *D. pastinaca* (Bacha et al. 2013). Similarly, skin mucus of *D. pastinaca* was observed from the center of the Mediterranean Sea (Fuochi et al. 2017). The antibacterial and antifungal activity was indicated for a concentration of  $16.50 \mu\text{g } \mu\text{l}^{-1}$  of skin mucus. The highest antifungal and antibacterial inhibition were estimated as 50% and 40.44%, respectively. Also, no inhibition activity was determined for gram + strains. In our study, a concentration of  $50 \mu\text{g } \mu\text{l}^{-1}$  of acidic mucus extracts from *D. pastinaca* showed a 99.99% reduction in bacterial strains except for *E. coli*.

The skin mucus of thirteen different fish species was tested for antifungal, antimicrobial, and cytotoxic activities of epidermal extracts (Hellio et al. 2002). In this study, in which different organic extracts were tested, it was observed that, especially depending on the given fish species, dichloromethane and ethanol extracts had antimicrobial effects without any cytotoxic properties. In another study, the skin mucus of *Himantura gerrardi* and *D. sephen* was examined to inhibit different pathogens with acetic extracts (Vennila et al. 2011). The antimicrobial activities of the aquatic and organic extracts from the skin secretion of ballan wrasse (*Labrus bergylta*) caught in Turkish territorial waters were examined in Turkish Seas (Katra et al. 2016). Studies reveal that mucus extracted with different solvents may show different effects depending on the type of solvent and mucus ingredient. Within the scope of our study, apart from acid extract, crude extract, and organic extract were also examined, but the antibacterial effect could not be obtained in them (data not shown).

In our study, the mucus was extracted with an acidic solvent to obtain the basic antimicrobial compounds, such as peptides. Similarly, previous reports on acidic mucus extraction methods for *G. morhua*, *S. solea*, *D. sephen* and *Himantura gerrardi* demonstrated broad spectrum antibacterial activities (Hellio et al. 2002, Bragadeeswaran et al. 2011, Vennila et al. 2011). Referring to Kumari et al. (2011), antibacterial activities of freshwater fish species *Rita rita* and *Channa punctatus* were observed. Also, *Cyprio carpio*, *Pelteobagrus fulvidraco*, *Ctenopharyngodon idella* are the other examples of freshwater fish discovered to possess novel antimicrobial compounds (Asakawa 1970, Lemaitre et al. 1996, Shen et al. 2012).

The antibacterial compounds as proteinases were purified from various fishes such as trout, salmon, and eel. In literature, pardaxin by *Pardachirus marmoratus*

and pleurocidin by the winter flounder *Pleuronectes americanus* were displayed as examples. Another important compound detected in the skin mucus secretions of cartilaginous fish is pentraxin, a type of lectin from *Raja kenoeji* (Tsutsui et al. 2009). All these compounds displayed small molecular weight properties. The higher molecular weight peptides could also be found in the epidermal mucus of carp and catfish (31 kDa, 30kDa, respectively) (Lemaitre et al. 1996). Here, we reported three different subunit bands, from the skin mucus of batoid species, between 10 kDa and 100 kDa. However, we did not identify these subunits.

The studies regarding the observation of antimicrobial properties of the skin mucus of marine fishes are limited in comparison to the studies of the skin mucus of freshwater fishes. However, the preliminary assays of skin mucus in the study indicated that the marine fishes are important sources for developing potential antimicrobial compounds against the invading pathogens in the absence of evident toxic effects on mouse fibroblasts. Further research needs to focus on the identification of specific compounds responsible for new pharmacological strategies for antimicrobial activity. It might show alternative usage as the potential by-product of wild marine sources for different industries. The skin mucus of marine species in the seas around Turkey can be an interesting source for new antimicrobial compounds.

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