Antibacterial Activity of Epidermal Mucus from Scarus Species

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Abstract

The antibacterial activities of epidermal mucus in crude extract from *Scarus dimidiatus, Scarus bleekeri,* and *Scarus forsteni* against three pathogenic bacteria (*Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*) were tested by determining the zone of inhibition and Minimum Inhibitory Concentration (MIC). Stock cultures of the microorganisms were sub-cultured and inoculated into Mueller Hinton Agar plates with mucus-impregnated filter paper. The diameter of each zone of inhibition was measured after 16-18 hours of incubation. Gram Staining was also performed, and bacterial cultures were diluted into 4-fold steps. A total of 0.2 ml of mucus was inoculated into Mueller-Hinton Broth to determine the MIC. Test for significance of difference demonstrated that S. forsteni exhibited the largest average zones of inhibition, followed by *S. dimidiatus* and *S. bleekeri. P. aeruginosa* was the most susceptible to fish mucus, followed by *E. coli* and *S. aureus*. Gram staining results suggest that the three bacteria were successfully inhibited, but the bacteria intrinsically present in fish mucus were not. None of the tubes turned clear for the measurement of the MIC. The turbidity observed was most likely due to 1) the presence of other bacteria inherently present, 2) the mucus used was not purified, and 3) the mucus was already turbid. With these findings, it can be concluded that the epidermal mucus of *S. dimidiatus, S. bleekeri,* and *S. forsteni* inhibits the growth of *E. coli, P. aeruginosa,* and *S. aureus* and thus plays an important role in fish against invading pathogens.

Keywords: Antibacterial, Fish epidermal mucus, Parrotfish

Introduction

Changes in the environment over the past years may be attributed to the growing trend of industrialization and urbanization in global communities and the accumulation of waste products due to the

commercialization of society (Denamur & Matic, 2006). These particular human activities cause an increase in the number of disease-causing pathogens and the incidence of mutations in such organisms affecting flora, fauna, and most notably, human life (World Health Organization, 2012). As a result, there is a continuing need to develop more drugs that could specifically target these pathogens and their mutant forms (Schunk et al., 2006).

Commercial pharmaceutical companies are continually involved in the development of new drugs. Although these new drugs have proven their efficiency in inhibiting the growth of common disease-causing pathogens (Denamur & Matic, 2006), these drugs are often priced in a way that the general Filipino public would find expensive.

To address this problem, non-profit and government-based medical research institutions worldwide are conducting studies involving sustainable and cheap sources of alternative medicine (particularly antimicrobials) and their practicability. One of the most sought-after topics in scientific research today is finding a sustainable source of drugs for treating disease-causing pathogens and other diseases that have no known cure yet. By the word sustainable, it means organic and ecological. Most, if not all, sustainable sources utilized by research today are plant and animal sources. Marine life is one.

In fish, the epidermal mucus is one of the key components of innate immunity. Previous studies (Kimbrell & Beutler, 2001) have shown that mucus secreted by fish significantly inhibits parasite, bacteria, and fungi colonization (Kuppulakshmi et al., 2008).

Epidermal mucus is characterized by a slimy and clear fluid layer free from protozoa, fungi, or other viable cells. Previous studies have shown that mucus secreted from fish play a significant part in the inhibition of parasite, gram positive and gram negative bacteria (al Arifa et al., 2011), and fungi colonization (Kuppulakshmi et al., 2008), suggesting their possible contribution to the advancement of therapeutic medicine (Haniffa et al., 2009). In a study by Loganathan et al. (2011), the collection of fish mucus samples in Clarias batrachus (in a saline medium) showed a significant activity against both the Gram-positive and Gram-negative bacteria. In another study by Kuppulakshmi et al. (2008), strong inhibition against the growth of tested bacteria was observed using Channa punctatus and Cirrhinus mrigala mucus extract.

One locally thriving fish in the Philippines is the Parrotfish, usually found in shallow reefs. There are approximately 34 Parrotfish species in the Philippines, three of which are Scarus dimidiatus, Scarus bleekeri, and

Scarus forsteni (Broad, 2003). Although studies are available on the antibacterial activity of some fish, to date, no studies have been done on parrotfish yet. Hence, in the present study, the antibacterial activities of fish epidermal mucus isolated from Scarus dimidiatus, Scarus bleekeri, and Scarus forsteni against three pathogenic bacteria, viz., Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus, are investigated.

Materials and Methods

A. Pre-Data Collection

The fish samples were collected from a local market in Dumaguete City. The fish were then selected through convenience sampling, taking note of their species. Pictures of the selected Parrotfish were then sent to the Silliman University Marine Laboratory to identify and confirm the species.

B. Collection of Mucus and Preparation of Crude Extract

Following the procedure of Kuppulakshmi et. al. (2008), the mucus was collected through careful scraping of the dorsal body of the fish using a sterile wire loop. Care was taken in the collection process such that the ventral side of the fish did not come in contact with the spatula to avoid intestinal and sperm contamination. The collected mucus was then pooled, brought to the laboratory, and refrigerated at 40 °C to preserve its freshness and prevent bacterial growth and degradation.

After thawing the refrigerated samples, crude extract and pure fish mucus were prepared by centrifuging at 5000 rpm for 10 minutes. The supernatant obtained was then stored in a refrigerator at 4 °C as recommended in the study of Ong Yeong Wei et al. (2010).

C. Culture of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* and Impregnation of Mucus Samples

Stock cultures of the test microorganisms were subcultured into Nutrient Agar tubes. The freshly-prepared suspensions, which will be used as inocula, were exponentially grown from these overnight tube cultures. Five to eight colonies of the organism from the freshly-prepared suspensions were transferred into test tubes containing Trypticase Soy Broth. The tubes were then incubated for 2-4 hours at 37 0C. The densities of the inocula for the three cultured bacteria were compared to that of a standard (0.5%

MacFarland's Standard). Having the same turbidity as MacFarland's Standard, swabs saturated with the inocula were inoculated into Mueller-Hinton Agar plates using the multiple overlap streaking method, leaving no area of the plates unswabbed. Mahon, Lehman, and Manuselis adopted this method.

The plates inoculated with the bacteria were then placed with sterilized circle-shaped mucus-impregnated filter paper discs using sterile forceps. The discs were placed on the agar, 10 mm apart, and were immediately covered and incubated for 16-18 hours at 370C under aerobic conditions. The same method was also done for the positive control using Ampicillin, Penicillin, and Gentamicin for *E. coli, S. aureus,* and *P. aeruginosa*, respectively (Loganathan et al., 2011) as well as the negative control. Replication was done three times for each sample to ensure the results' validity and reliability. All plates were incubated for 16-18 hours at 37 0C under aerobic conditions.

D. Measurement of the Zone of Inhibition and Gram Staining

After 16-18 hours of incubation, the diameter of each zone of inhibition for each filter paper in each plate was measured. The plates were placed a few inches above a black, non-reflecting surface, and the zones were examined from the back side (where the agar is) of the plate, illuminated with reflected light.

Gram staining was immediately performed after measuring the zones of inhibition. Three drops of sterile water were placed on the slide. Inoculating a small amount of bacterial growth from the plate was performed by slightly touching the colony's surface, which was then emulsified into sterile water. After which, the smear was air-dried by passing the slide rapidly over the flame with the smear side up 2-3 times. The Gram Staining procedure was adapted from the book *Diagnostic Microbiology* by Mahon et al. (2010).

E. Measurement of Minimum Inhibitory Concentration (MIC)

Freshly-prepared suspensions were again obtained from overnight culture tubes and were transferred into test tubes containing Mueller-Hinton Broth. The concentration of the bacterial suspension was adjusted to 108 colony-forming units (108 CFU/mL) in Mueller Hinton Broth (Ong et al., 2010). Again, for visual comparison, a 0.5% McFarland standard was utilized (ESCMID, 2000). The tubes containing the cultured bacteria inoculated with 108 CFU/mL were diluted in 4-fold steps. With each successive dilution, the concentration was reduced by half.

In determining MIC for this particular study, 0.2 mL of the crude mucus treatment was delivered into the tubes as indicated in Subramanian, Ross, and Mackinnon's study and incubated at room temperature for 16 to 18 hours. The test tubes in the series were examined for growth by observing the clarity or turbidity of the broth.

Table 1

Antibacterial Activity of the Mucus of Three Species of Parrotfish against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Positive and Negative Controls as Measured in Zone of Inhibition (mm)

Treatment	Average Zone of Inhibition (mm)		
	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Scarus dimidiatus	18.67	20.67	15.33
Scarus bleekeri	18.00	17.67	11.33
Scarus forsteni	21.67	22.67	17.33
Positive Control (Ampicillin)	17.62		-
Positive control (Gentamicin)	-	23.33	-
Positive Control (Penicillin)	-	-	27.33
Negative Control	0	0	0

Results

A. Zone of Inhibition

Table 1 shows the zone of inhibition for each species of Parrotfish, for each bacterium, and for positive and negative controls after 16-18 hours of incubation. The mucus from all three Parrotfish species inhibited the growth of the three bacteria. Based on the data, S. forsteni recorded the largest zone of inhibition against all bacteria among the three fish species. Among the three bacteria, it is most potent against *P. aeruginosa.*

In addition, *S. dimidiatus* recorded the largest zone of inhibition in *P. aeruginosa,* while *S. bleekeri* recorded the largest zone of inhibition in *E. coli.* It is also noteworthy that for *E. coli,* all three species of Parrotfish yielded a zone of inhibition larger than the positive control (Ampicillin).

A test for the significance of the difference was also performed to assess whether there is a difference in the potency of epidermal mucus in bacterial growth inhibition among the three species of Parrotfish and three bacteria.

Table 2

Test for the Significance of the Difference in the Isolated Fish Epidermal Mucus

Source of Variation or Difference	f-test statistics			Interpretation (α= 0.05)
	f-computed	f-tabular	p-value	
Different species of Parrotfish	57.126	3.008	0.000	Significant
Different types of bacteria	56.736	3.403	0.000	Significant
Interaction (Antibacterial Activity)	17.088	2.508	0.000	Significant

Table 2 shows that there is a significant difference in the zone of inhibition of the isolated epidermal mucus among the three species of Parrotfish. The positive control made the largest zone of inhibition, followed by *S. forsteni, S. dimidiatus*, and *S. bleekeri*. The data also suggest that S. forsteni collectively has the same ability as the positive control regarding bacterial inhibition, while *S. dimidiatus* and *S. bleekeri* are also comparatively the same.

The same table also showed that there is a significant difference in the zone of inhibition of the isolated epidermal mucus among the three types of bacteria. P. aeruginosa was the most susceptible to the epidermal mucus, followed by E. coli and S. aureus. A significant interaction (antibacterial activity) was observed between the different species of Parrotfish and the different types of bacteria. Table 1 shows that Penicillin had the highest antibacterial activity in the positive control against S. aureus. On the other hand, the lowest antibacterial activity was seen in *S. bleekeri* against *P. aeruginosa.*

Finally, data for Duncan's Multiple Range Test at a 0.05 significance level is shown in Table 3. The results imply no significant difference in the inhibition zones among the first three combinations and combinations four to seven.

Table 3

Ranking of Zones of Inhibition as a Result of the Interaction of Crude Mucus Collected from Different Species of Parrotfish against Different Types of Bacteria

Combination of Parrotfish species and Type of Bacteria	Zone of Inhibition (Mean)	Rank
1. Scarus forsteni & Staphylococcus aureus	22.6667	2.0
2. Scarus forsteni & Escherichia coli	21.6667	2.0
3. Scarus dimidiatus & Staphylococcus aureus	20.6667	2.0
4. Scarus dimidiatus & Escherichia coli	18.6667	5.5
5. Scarus bleekeri & Escherichia coli	18.0000	5.5
6 Scarus bleekeri & Staphylococcus aureus	17.6667	5.5
7. Scarus forsteni & Pseudomonas aeruginosa	17.3333	5.5
8. Scarus dimidiatus & Pseudomonas aeruginosa	15.3333	8.0
9. Scarus bleekeri & Pseudomonas aeruginosa	11.3333	9.0

B. Gram Staining

Gram staining was performed to assess whether other bacteria, besides those tested, were inherently present in fish mucus. This was done after the zones of inhibition observed were not completely clear and translucent, which may imply that the bacteria were not completely inhibited. Table 4 shows the Gram staining results after 16-18 hours of incubation.

Table 4

Gram Staining Results of Antimicrobial-impregnated Discs of the Different Species of Parrotfish Measured in Terms of Zone of Inhibition against the Different Bacteria

Fish Species	Gram Stain Reaction		
	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Scarus dimidiatus	Gram positive rods	Gram positive rods	Gram positive rods
Scarus bleekeri	Gram positive cocci	Gram positive cocci	Gram positive rods
Scarus forsteni	Gram negative rods Gram positive cocci	Gram positive rods	Gram positive rods

C. Minimum Inhibitory Concentration

The measurement for the MIC was done right after Gram Staining. After the entire procedure, none of the tubes were clear after 16-18 hours of incubation.

Discussion

Epidermal mucus is found on the epithelial surface layer of fish. It acts as a physical and chemical barrier (Floyd, 2009). Antimicrobial compounds, incessantly secreted and cast off the skin of fish through its mucus (Ellis, 2001), are usually produced by the granular glands of amphibians and are linked with and diffused from the epithelial mucus-secreting cells of fish (Kuppuulakshmi et al., 2008).

The number of mucus-producing cells in the epidermal and epithelial layers, the exposure of fish to disease-causing microorganisms, and environmental agitation influence the amount of mucus produced among different fish species (Subramanian et al., 2008). The quantity of mucus secreted in different fish species has been found to play a part in the fish's vulnerability to certain infections. Fish with fewer or no scales produced more elevated amounts of epidermal mucus than fish with scales (Pickering, 1974). Moreover, the biochemical substances found in mucus differed depending on the ecological and physiological conditions where the

fish are found (Subramanian et al., 2008).

Epidermal mucus specifically contains specific and non-specific antimicrobial compounds and complement factors. These lysozymes degrade bacterial cell walls (Rakers et al., 2010), proteases, C-reactive protein, lectin-like molecules, agglutins, and glycoproteins (Caipang et al., 2011).

In addition, peptides and proteins in fish mucus exhibit a broad spectrum of antimicrobial activities against various fish and human pathogens (Gobinath et al., 2011). Peptides are thought to employ different strategies: fatal depolarization of the cell membrane, formation of pores, and subsequent leakage of the cell contents and damage of critical intracellular targets after internalization of the peptide (Balasubramanian et al., 2011).

The present study tested the antibacterial activity of epidermal mucus secreted by S. forsteni, S. dimidiatus, and S. bleekeri in crude extract against three common human pathogens-- *E. coli, P. aeruginosa*, and *S. aureus*. Based on the data obtained, *S. forsteni* recorded the largest zone of inhibition against all bacteria among the three fish species. Among the three bacteria, it is most potent against *P. aeruginosa.*

In a study by Loganathan et al. (2011) on the role of mucus from *Clarias batrachus* against selected microbes, *P. aeruginosa* was also observed to have the largest zone of inhibition, which was about 25 mm in diameter, when compared to other bacteria such as *Aeromonas hydrophila, Vibrio anguillarum, Vibrio fischeri,* and *Escherichia coli.* Similar findings have also been reported by Dhanaraj et al. (2009), wherein the maximum antibacterial activity (largest zone of inhibition) was observed in the intestinal mucus of all five *Channa spp.* against *P. aeruginosa.* According to Mahon et al. (2010), *P. aeruginosa* is ubiquitous in soil and water, on surfaces in contact with soil or water, and rarely found in the human microbiota. This explains why *P. Aeruginosa* exhibits the largest zone of inhibition because it more often comes in contact with fish and other marine fauna than the other bacteria in this study, thus increasing the fish mucus' resistance against it.

In addition, *S. dimidiatus* recorded the largest zone of inhibition in P. aeruginosa, while S. bleekeri recorded the largest zone of inhibition in E. coli. According to Loganathan et al. (2011), the cells that produce mucus in epithelial and epidermal layers have been reported to vary between fish species and therefore could influence mucus composition. The biochemical substances of mucus have been proven to vary depending on the physiological and ecological conditions such as pH, salinity, handling stress, and stages of growth and maturity.

The results for Duncan's Multiple Range Test at the 0.05 level of significance imply no significant difference in the inhibition zones among

the first three combinations and combinations four to seven. This is consistent with the findings in a study by Loganathan et al. (2011) wherein the mucus collected from *Clarias batrachus* showed a significant activity regarding the Gram-positive and Gram-negative bacteria.

As for Gram staining, the results generally suggest that the three bacteria in this study were successfully inhibited by fish mucus. However, the other bacteria present, which are contaminants intrinsically present in fish mucus, were not. Although the findings in a study by Loganathan et al. (2011) showed a significant activity with regards to Gram positive as well as Gram negative bacteria, the bacteria which are intrinsically present in fish mucus may serve as normal flora i.e., non-pathogenic to the fish, therefore the antibacterial properties found in fish mucus are not bactericidal to these particular bacteria. Examples of normal flora found in fish are *Bacillus spp*.— a Gram--positive rod, *Streptococcus spp*.— a Gram positive cocci (Olojo et al., 2002), *Enterobacter aerogenes*— a Gram-negative rod, and *Aeromonas hydrophila*— also a Gram-negative rod.

As for the minimum inhibitory concentration, the turbidity observed in all the tubes are most likely due to 1) the presence of other bacteria inherently present in fish mucus as was seen during Gram staining, 2) the fish mucus used was not purified therefore increasing the likelihood of contaminants; and 3) the fish mucus in itself was already turbid.

In other studies, acidic, aqueous, and organic extracts were also used apart from the crude extract utilized in the present study. However, only the acidic extracts were found to have a broad range of activity against the pathogens. All the other extracts, i.e., crude and organic, proved otherwise. The results of the present study showed that the epidermal mucus of Scarus dimidiatus, *Scarus bleekeri*, and *Scarus forsteni* inhibits the growth of three pathogenic bacteria-- *Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus,* and thus plays an important role in fish against invading pathogens. The results imply that fish mucus could thus be regarded as an alternative antibacterial agent against common pathogens such as the three bacteria tested in this study.

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