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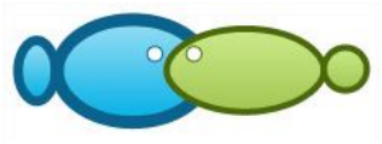

Antibacterial activity of the epidermal mucus of Nile tilapia (*Oreochromis niloticus*) against pathogens in vannamei shrimp (*Litopenaeus vannamei* Boone)

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Abstract. Shrimp production is affected by disease attacks caused by various types of pathogenic *Vibrio* species. Mucus secreted in the epidermis of the fish skin acts as the first line of defence between fish and pathogens in their living medium. The purpose of this study was to evaluate the antimicrobial potential of Nile tilapia (*Oreochromis niloticus*) epidermal mucus against pathogenic bacteria in white shrimp (*Litopenaeus vannamei* Boone). Crude, acid, and aqueous mucin extracts of the epidermis were prepared, and their antibacterial activity was tested by disc diffusion method against three bacterial pathogens of vannamei shrimp; *Vibrio harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*. The antibacterial activity was measured in the zone of inhibition in mm and compared with the antibiotic Streptomycin as a positive control, distilled water, and acetic acid as negative controls. Of the 27 tests performed (three types of epidermal mucin extract against three different bacterial strains with three repetitions), 15 tests showed antibacterial activity. Acid extracts showed inhibitory potential against pathogenic bacterium *V. harveyi,* crude and acidic extracts were able to inhibit *V. parahaemolyticus*, while *V. alginolyticus* could be inhibited by the three types of epidermal mucin extracts. *O. niloticus* acid extract showed inhibitory activity for all types of test pathogens; *V. harveyi, V. parahaemolyticus*, and *V. alginolyticus*. The acidic extract of *O. niloticus* significantly affected *V. harveyi, V. parahaemolyticus,* and *V. alginolyticus* at a minimum concentration of 20 µL mL^{-1}, but the aqueous extract of *O. niloticus* required 60 µL mL^{-1} of protein to inhibit *V. harveyi*. *O. niloticus* epidermal mucin can be a natural product, which can help overcome the problem of antibiotic resistance of many pathogenic bacteria in shrimp culture.

Key Words: acid extract, aqueous extract, crude extract, epidermal mucin, tilapia, vannamei.

Introduction. Vannamei shrimp (*Litopenaeus vannamei* Boone) is one of the leading shrimp species aquaculture in the world (Manoppo et al 2011). In 2018, global production of shrimp was more than 4.5 million tons, and 77% of that production were vannamei shrimp (Anderson et al 2016). Indonesia has become one of the main exporters to the US market, sharing the US market with India, the exports of the two countries accounted for 44% of US imports in 2016 (Anderson et al 2016). The characteristics possessed by this shrimp species are the reason to use it as a leading commodity for cultivation. Vanname shrimp has many advantages, including being relatively resistant to disease, growing relatively fast, being able to utilize space more efficiently, and being more tolerant to environmental changes (Jiburba 2012; Schock et al 2013; Maicá et al 2014).

However, the progress of shrimp farming cannot be separated from disease attacks. The disease is the main obstacle to increasing cultivation production because it can cause relatively high mortality. Disease attacks caused Indonesian shrimp farming export volume to decrease by 64.9 thousand tons in 2018 to 62.64 thousand tons in 2019 (KKP, JIKA 2017). One type of disease that can cause mass mortality in cultured

shrimp is vibriosis. This disease is caused by bacteria of the *Vibrio* genus such as *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus* (Asplund 2013; Nor-Amalina et al 2017), and infection with these various types of *Vibrio* species occurs at every stage of shrimp development, starting from the hatching stage, seed rearing to stocking in the grow-out pond (de Souza Valente & Wan 2021).

Matsumoto et al (2000) reported that *V. harveyi* was one of the causative agents of mass mortality during the rearing of Penaeid larvae. Disease attacks occur at all stages of life but are more common in hatcheries. Kumar et al (2021) reported that *V. parahaemolyticus* is a halophilic bacterium distributed in temperate and tropical coastal waters throughout the world. Some types may cause acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) with a very high mortality rate and cause economic losses. *V. alginolyticus* has been isolated from the sick shrimp, showing poor growth, anorexia, inactivity, redness of uropods and telsons, muscle blurring, and death. *V. alginolyticus* is a Gram-negative rod-shaped bacterium. Histological preparations of the infected shrimp with bacteria showed melanized hemocytic granulomas occurring in the connective tissue around the hemal sinus and haemocyte aggregation in necrotic muscles (Liu et al 2004).

Until now, antibiotics are still widely used to fight against bacterial infections in the aquaculture industry. For example, oxytetracycline, tetracycline, florfenicol, and oxolinic acid are mainly used to control vibriosis in shrimp. Quinolones and flumequine are widely used to treat cold vibriosis caused by *Vibrio salmonicida* and classical vibriosis caused by *Vibrio anguillarum*. However, resistance to antibiotics has been found in some *Vibrio* spp. bacteria due to the overuse of antibiotics in treating diseases in the aquaculture industry (Cabello et al 2013).

This problem has received significant attention due to the increasing resistance of pathogens *V. parahaemolyticus, V. harveyi, V. alginolyticus,* and *V. vulnificus* to many antibiotics circulating in the market and used clinically (Kang et al 2017). In addition, some antibiotic resistance causes severe economic losses to the aquaculture industry (Zhu et al 2017). Extensive use of antibiotics promotes the development of antibiotic resistance and contaminates food, water, and sediment (Kraemer et al 2019). Defoirdt et al (2011) reported the increasing use of antibiotics such as chloramphenicol, cotrimoxazole, erythromycin, and streptomycin to control the *Vibrio* infection in tiger prawns (*Penaeus monodon*), causes bacteria to become resistant, and antibiotics are no longer effective.

According to Prajitno (2007), it is better to overcome the disease in shrimp; an alternative treatment is needed using natural ingredients derived from plants and aquatic biota. One that can be used is epidermal mucus on fish skin. Fish skin epidermal mucus is a stable physical or chemical barrier against pathogens. In fish, the surface of the epithelial tissue is covered by a smooth layer of epidermal mucus. Epidermal mucus is a thick colloidal substance that contains antibacterial enzymes, protein and water, and several other substances known as mucus. Mucus serves as an essential component of the innate immune mechanism, which is continuously secreted or exfoliated regularly, to prevent the attachment of pathogens and colonization of disease-causing microbes and parasitic invasion (Arasu et al 2013). Arockiaraj et al (2012) reported that fish epidermal mucus contains several innate immunity factors such as proteins and enzymes like lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein (CRP), proteolytic enzymes, alkaline phosphatase (ALP) and various antimicrobial peptides.

The antimicrobial potential of fish epidermal mucus is highly dependent on the fish species and habitat (Subramanian et al 2008). Mucus in fish skin epidermis also varies significantly in viscosity, thickness, and content of glycoprotein (mucus) which is also the main component of fish mucus and depends on the fish species (Dash et al 2018). In addition, the extraction method used also affects the antibacterial potential of fish epidermal mucus. Wei et al (2010) stated that acid extraction of epidermal mucus showed more excellent inhibitory activity against pathogenic bacteria in humans and fish than aqueous and crude extraction.

From this description, it is urgent to determine the method of extracting the epidermis mucus of Nile tilapia (*Oreochromis niloticus*), which provides the most

significant antibacterial potential in inhibiting the growth of *V. harveyi, V. parahaemolyticus*, and *V. alginolyticus* bacteria, and to evaluate the antibacterial potential of Nile tilapia epidermal mucus in a challenge test against pathogenic bacteria *V. harveyi, V. parahaemolyticus* and *V. alginolyticus*.

Material and Method

Fish mucus collection and extraction. This research was carried out from July to October 2021 at the Fish Health Laboratory, Department of Aquaculture Technology, Pangkep State Polytechnic of Agriculture, South Sulawesi, Indonesia. Samples of Nile tilapia (both male and female) aged about 1.5-2 months and weighing approximately 200-grams were obtained from freshwater fish farming ponds in Pinrang Regency, South Sulawesi Province. The fish obtained were then acclimatized for a week in the fish tank. During this period, the fish were fed commercial diets once daily on an ad libitum basis. Every day 20-30% of water changes were carried out. After one week of acclimatization, the fish were ready to be used for taking epidermal mucus. Only healthy fish were selected for epidermal mucus collection. Dead fish and fish with abrasions or lesions were removed from the rearing tank. Fish rearing tanks were cleaned daily to maintain optimal water quality and to avoid microbial infections.

Before taking the mucus, the fish have fasted for 24 hours. A number of 20 fish were put into a plastic container with a volume of 40 liters followed by 50 mL of 50 mM NaCl into the container. The plastic container was gently shaken for 5 minutes to trigger stress and mucus discharge on the surface of the fish skin. Secreted mucus was collected using a 15 mL centrifuge tube. The collected mucus was immediately centrifuged for 10 minutes at 1500 rpm at 4°C. The supernatant was transferred to a new centrifuge and then stored in a freezer at -20 °C until analyzed (extract stock). When mucus samples were collected, anesthetic chemicals were not used. The supernatant was divided into three parts and extracted separately by crude, acidic, and aqueous methods.

Crude extraction. Crude extraction was carried out based on the Subramanian et al (2008) method with some modifications. About 250 mg of mucus extract in freeze-dried form were resuspended in 10 mg mL^{-1} of water and centrifuged at 9500 × g (Beckman coulter, Avanti J-26 XPI) for 10 min at 4°C. The supernatant was collected and stored at -4°C until further antimicrobial testing.

Aqueous extraction. Aqueous extraction was carried out according to Subramanian et al (2008) with some modifications. About 250 mg of the mucus supernatant in freeze-dried form was resuspended in 100mM (w/v) ammonium bicarbonate at (1 mg mL^{-1}) and homogenized using a vortex. Next, the homogenized suspension was centrifuged at 30,000 g for 30 min at 4°C. The supernatant was collected and filtered through Whatman no. 1 filter paper and continued with 0.45-micron Whatman nylon filter. The filtrate was stored at 4°C (Jothi et al 2014) until further antimicrobial testing.

Acidic extraction. According to Hellio et al (2002) and Conlon (2007), acid extraction was carried out with some modifications. About 250 mg of the lyophilized mucus supernatant added to 1% acetic acid (HAc) moderate (1:4) was placed in a boiling water bath for 3 minutes to inhibit the activity of the proteolytic enzymes. The heated mixture was homogenized using a vortex (on ice) for 1 minutes. The resulting homogenate was centrifuged in a refrigerated centrifuge at 25,000 g for 35 minutes, at 4°C. A volume of 600 mL of the homogenate supernatant was filtered through Whatman no. 1 filter paper, followed by a 0.45-micron Whatman nylon filter. The filtrate was stored at 4°C until further antimicrobial testing.

Minimum inhibitory concentration (MIC) test. The purpose of the MIC test was to determine the lowest concentration of epidermal mucus capable of inhibiting the growth of *V. harveyi, V. parahaemolyticus* and *V. alginolyticus* according to the method recommended by Dahak & Taourirte (2013) with some modifications. MIC was performed

by serial dilution of the three extracts in six concentrations of 10, 20, 30, 40, 50, and 60 µL mL^{-1}. Bacteria with a density of 2 × 10^6 were grown in Mueller Hinton broth and incubated at 37°C. MIC was determined as the lowest concentration required to inhibit microbial growth. All tests were conducted in triplicate, using 2% DMSO as a negative control.

Preparation of bacterial test. The bacteria used were *V. harveyi* (MP1), *V. parahaemolyticus* (SB28), and *V. alginolyticus* (BJ23) from the collection of the Research Institute for Brackish Water Aquaculture and Fisheries Extension (BBPBAP3) Maros, South Sulawesi. The bacterial isolates were first inoculated separately into the TCBS agar medium. Furthermore, each bacterial isolate was taken aseptically using a 0.1 mL micropipette and then inoculated separately into LB broth media using the spread-plate method (Pelczar et al 1993), then incubated on an orbital shaker for 24-h. Then it was measured using a spectrophotometer (Thermo scientific Genesys 20) (625-650 nm) to a density of 10^6 CFU mL^{-1}.

Antibacterial activity test. The antibacterial activity of epidermal mucus extract of Nile tilapia was tested using the Agar Diffusion method (Loh et al 2014). About 0.5 µL of pathogenic bacteria isolate with a density of 106 CFU mL^{-1} was cultured on Tryptic Soy Agar with the addition of 2% NaCl (w/v). After the media containing the pathogenic bacteria cultures solidified, paper discs (2 mm, Toyo Roshi Kaisha Ltd, Japan) were dripped with each epidermal mucus extract and 0.5 mL of Streptomycin ® 100 mg antibiotic as a positive control. In contrast, acetic acid and dH_2O as negative controls were performed in 3 replications and then incubated at 30°C for 24-h. Determination of antibacterial activity was performed by measuring the diameter of the clear zone formed around the paper disc. The filtrate containing antibacterial substances will inhibit pathogenic bacteria, as evidenced by the clear zone around the paper disc.

Data analysis. Data obtained during the study were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, which was used to determine significant variation between the epidermal mucus of the three test species and the antibiotics used. The test was used to determine the significant difference between the zone of inhibition of bacteria (zone of inhibition, ZOI) obtained from the third type of extract; crude, aqueous, and acidic obtained from three species of fish tested against the three types of pathogenic shrimp bacteria. Significant data were analyzed at a probability value of p 0.05. All data were analyzed using SPSS Version 22.

Results and Discussion

Antibacterial activity test. Nile tilapia epidermal mucus was extracted crude, aqueous, and acidic and its antibacterial activity against three strains of pathogenic bacteria in whiteleg shrimp was evaluated. Of the 36 tests performed, 18 tests showed antibacterial activity. The inhibitory potential of crude, aqueous, and acidic extracted Nile tilapia epidermal mucus is presented in Table 1. Nile tilapia epidermal mucus showed the maximum bactericidal effect (1.22±0.2 mm) against *V. alginolyticus*. Different things appear in the inhibitory effect of antibiotic Streptomycin, where the resistance of the bacteria *V. alginolyticus* to this antibiotic is characterized by the absence of a clear zone around the paper disc being dripped with Streptomycin. The same thing was seen in the negative control treatment, both using distilled water and acetic acid. Crude extracted epidermal mucus did not show any inhibitory effect against *V. harveyi* pathogen. Different things were seen in the inhibitory effect of crude extracted epidermal mucus against the pathogenic bacterium *V. parahaemolyticus* (1.07±0.08). Resistance of the three types of pathogenic bacteria to Streptomycin antibiotics was seen during this study, where the antibiotic found no zone of inhibition.

The aqueous extracted Nile tilapia epidermal mucus showed a zone of maximum inhibition (0.83±0.06 mm) against the pathogen *V. alginolyticus*. The epidermal mucus did not show any inhibitory effect against *V. parahaemolyticus* as shown by Table 1. The

same thing was observed with the use of the antibiotic Streptomycin, where it became increasingly clear that *V. parahaemolyticus* was resistant to Streptomycin. The inhibitory potential of aqueous extract was confirmed accurately by negative control treatment using either distilled water or acetic acid, where no zone of inhibition was seen in the challenge media.

The inhibitory potential of the acid-extracted Nile tilapia epidermal mucus showed the maximum inhibitory effect (2.30±0.01) against *V. harveyi* (Table 1). The same thing was observed in the inhibitory effect of epidermal mucus extract against *V. alginolyticus* bacterium. Epidermal mucus extract was also found to inhibit the growth of *V. parahaemolyticus* (1.06±0.05). When viewed from the source of epidermal mucus, the epidermal mucus sourced from Nile tilapia extracted with acid showed a more substantial inhibitory effect against pathogenic bacteria *V. harveyi* and *V. alginolyticus* than crude and aqueous extracts.

Table 1

The zone of inhibition (diameter) of crude, aqueous, and acidic extracts of *O. niloticus* against three types of bacterial pathogens

Mucus extract	*Diameter of the zone of inhibition (mm)*		
	V. harveyi	*V. parahaemolyticus*	*V. alginolyticus*
1. Crude extract of *O. niloticus*	-	1.07±0.08[b]	1.22±0.02[c]
2. Streptomycin	-	-	-
3. Acetic acid	-	-	-
4. dH_2O	-	-	-
1. Aqueous extract of *O. niloticus*	-	-	0.83±0.06
2. Streptomycin	-	-	-
3. Acetic acid	-	-	-
4. dH_2O	-	-	-
1. Acidic extract of *O. niloticus*	2.30±0.01	1.06±0.05	1.28±0.19
2. Streptomycin	-	-	-
3. Acetic acid	-	-	-
4. dH_2O	-	-	-

Note: Data are shown as the mean±SE. Different superscripts on the same row indicate statistically significant difference.

The antimicrobial screening results showed that no detectable level of antimicrobial activity was observed in crude and aqueous extracts of epidermal mucus against the pathogen *V. harveyi.* Filtration of crude and aqueous extracts from epidermal mucus inhibited the growth of the pathogen *V. alginolyticus*. These results suggest that antimicrobial components may be present in the epidermal mucus of tilapia. Previous studies have also reported that no inhibition of microbial growth was observed in aqueous extracts of epidermal mucus of a wider variety of fish species, including arctic char (*Salvelinus alpinus*), river trout (*Salvelinus fontinalis*), koi fish (*Cyprinus carpio*), striped bass (*Morone saxatilis*), haddock (*Melanogrammus aeglefinus*) and hagfish (*Myxine glutinosa*) (Andrews 2005). Furthermore, the antimicrobial activities of the extracted epidermal mucus with acidic, organic, and aqueous solvents vary widely within and between fish species (Subramanian et al 2008). The absence of antimicrobial activity of aqueous extracts in this study could be due to low levels of enzymes (Subramanian et al 2008). It has been reported that epidermal mucus containing enzymes can also affect innate defence by activating genes encoding proteins such as antimicrobial peptides and complement proteins and thus may exert antimicrobial activity through indirect mechanisms. For example, cathepsin D and matrix metalloprotease are involved in producing the antimicrobial peptide, parasin I, as reported in the epidermal mucus of the catfish *Parasilurus asotus* (Cho et al 2002).

In this study, the acidic extract of the epidermal mucus of Nile tilapia could inhibit three types of pathogenic bacteria in vannamei, *V. harveyi, V. parahaemolyticus* and *V. alginolyticus*. A similar thing was reported by Garcia-Marciano et al (2019) that acidic

extract from Nile tilapia adapted from the sea could inhibit *V. parahaemolyticus*. It was further reported that *V. harveyi* was also inhibited by acidic and organic extracts. Another result showed that the acidic extract of the epidermal mucus of *Channa striata* could inhibit the growth of three human pathogens, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* but was unable to inhibit the fish pathogen *Aeromonas hydrophila* (Wei et al 2010). Kumari et al (2011) reported that the skin mucus of *Rita rita* and *Channa punctatus* extracted in acidic and aqueous solvents show antibacterial activity against *Staphylococcus aureus* and *Micrococcus luteus*. However, the activity of extract from the acidic solvents was higher than that from aqueous. The acidic mucus extracts of *R. rita* also show antibacterial activity against *Salmonella typhi*.

Furthermore, Subramanian et al (2008) reported that the acidic mucus extracts of brook trout, haddock and hagfish exhibit bactericidal activity against various fish and human pathogens (Subramanian et al 2008). Likewise, Hellio et al (2002) reported that *K. pneumoniae* growth was inhibited by fish mucus extract. Previous studies have shown that various antimicrobial proteins such as paradaxin and pleurocidin from fish epidermal mucus are protective functions against pathogens (Wei et al 2010).

Minimum inhibitory concentration (MIC) test. Different fish epidermal mucus extracts showed different MICs against the three microbial strains tested. Crude and acidic extracts from the fish samples showed a minimum inhibitory effect at a concentration of 20 µL mL^{-1} against *V. harveyi, V. alginolyticus*, and *V. parahaemolyticus* bacteria. While aqueous extraction showed a minimum inhibitory effect at a concentration of 60 µL mL^{-1} for the pathogen *V. harveyi* and 40 µL mL^{-1} for the other two types of pathogens; *V. parahaemolyticus* , and *V. alginolyticus* (Table 2).

Table 2

MIC of three types of tilapia mucus extracts against the three microbial strains

Pathogenic bacteria	*MIC (µL mL^{-1})*	*Mucus extracts*	*Zone of inhibition (diameter, mm)*
V. harveyi	20	Crude extract	1.05
	60	Aqueous extract	0.74
	20	Acidic extract	0.95
V. parahaemolyticus	20	Crude extract	1.16
	40	Aqueous extract	0.76
	20	Acidic extract	1.15
V. alginolyticus	20	Crude extract	0.95
	40	Aqueous extract	0.64
	20	Acidic extract	1.04

The results showed that concentrations of 20 µL mL^{-1} in the crude and acidic extract and 60 µL mL^{-1} in the aqueous extract inhibited the growth of *V. harveyi*. The minimum concentration of crude and acidic mucus extract was 20 µL mL^{-1} found to inhibit the growth of the shrimp pathogen *V. parahaemolyticus* . Meanwhile, aqueous mucus extract was found to inhibit *V. parahaemolitycus* at a concentration of 40 µL mL^{-1}. A similar trend was observed in *V. alginolyticus*, where the crude and acidic mucus extract was found to inhibit the growth of the shrimp pathogen at a concentration of 20 µL mL^{-1} while a higher concentration was 40 µL mL^{-1} for the aqueous extract.

Previous studies reported that several extracts of epidermal mucus had been found to inhibit the growth of *Escherichia coli* and *S. aureus*, and the MIC values observed were within the same range as the present study. Thus, the epidermal mucus fraction of eel (*Anguilla anguilla*), tench (*Tinca tinca*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), carp (*Cyprinus carpio*), winter flounder (*Pleuronectes americanus*), and moses fish (*Pardachirus marmoratus*) inhibited the growth of *P. aeruginosa*, *Pseudomonas fluorescens*, *E. coli*, *A. hydrophila* and *S. aureus* (Wei et al 2010). It was further described that the epidermal mucus of *C. striata* exhibited an inhibitory effect on several microorganisms. Likewise, acidic extract of the epidermal mucus of *C. striata* was examined to determine the MIC by agar-overlay

diffusion method and micro broth plate dilution. The results showed that C. striata showed the highest inhibitory effect against *P. aeruginosa* and *A. hydrophila* compared to a broad-spectrum control antibiotic, Cefoperazone. The catfish (*Clarias batrachus*) had the highest inhibitory activity since it inhibited two pathogens at a 1:4 concentration (Lirio et al 2018).

The effectiveness of inhibiting pathogenic bacteria by extracting mucus under acidic conditions was due to the higher ability of the protein to become more soluble (Hancock & Sahl 2006). They were further confirmed by Ming et al (2007), who showed that acidic solutions increase the solubility of cationic peptides responsible for antibacterial activity. The antibacterial activity of epidermal mucus may be due to the presence of antibacterial glycoproteins that could kill bacteria by forming large pores on the target membrane. Fish epidermis mucus is believed to play an essential role in preventing colonization by parasites, bacteria and fungi and thus acts as a chemical defence barrier.

Conclusions. This study investigated *Oreochromis niloticus* mucus extract (crude, aqueous and acidic) against three types of shrimp pathogens. From the results obtained, the acidic extract could inhibit the growth of three shrimp pathogens, *Vibrio harveyi, Vibrio parahaemolyticus,* and *Vibrio alginolyticus*. The crude extract and aqueous extract inhibited the growth of the shrimp pathogen *V. alginolyticus*. This study also suggested that *O. niloticus* mucus could be a potential source of antimicrobial activity for specific shrimp pathogens. In the future, further investigations could be focused on other shrimp bacterial pathogens.

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Conflict of interest. The authors declare that there is no conflict of interest.

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