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Screening of antibacterial activity of mucus extract of Snakehead fish, *Channa striatus* (Bloch)

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Abstract. – *Aim:* **The objective of this study is to gain a better understanding of the antimicrobial properties of the mucus extract of snakehead fish, *Channa striatus* against selected human and fish pathogenic microbes.**

Materials and Methods: **The fish mucus samples were extracted with crude, acidic and aqueous solvents to identify potential antimicrobial agents including aqueous and acid soluble compounds. The study also determined the protein content of the three different mucus extracts. The highest protein content (0.589 mg/ml) was noticed in the crude extract followed by aqueous mucus extract (0.291 mg/ml) and acidic extract (0.267 mg/ml). Preliminary screening for antimicrobial activity of all three mucus extracts were tested against 5 human pathogens (*Bacillus subtilis*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) and fish pathogen (*Aeromonas hydrophila*) using the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc susceptibility test method. The activity was measured in terms of zone of inhibition in mm.**

Results: **The acidic mucus extracts exhibited a bactericidal activity and inhibited the growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Bacillus subtilis* while aqueous and crude extract showed no bactericidal activity for any of the human pathogens tested. Further test against fish pathogen *Aeromonas hydrophila* showed that the aqueous and crude extracts are capable of inhibiting the growth of the pathogen, demonstrating the presence of antimicrobial agents and the role of fish mucus in antimicrobial protection.**

Conclusions: **The present results suggest that the mucus extracts of snakehead fish *Channa striatus* may be a potential source of antimicrobial agents for human and fish pathogens.**

Key Words:

Snakehead fish *Channa striatus*, Epidermal mucus, Antibacterial activity.

Introduction

Advanced improvements and new formulations in the modern chemotherapeutic techniques have been applied. However, infectious diseases are still an increasingly important public health issue in the world[1]. It has been reported that two million people died in 2000 due to diarrhoeal disease worldwide[1]. To combat this, recently research has been made to find out an effective method to prevent or cure diseases. Nowadays, the development of resistance to many of the commonly used antibiotics provides a further attempts to search for new antimicrobial agents to combat infections and overcome problems of their resistance and side effects. Several attempts have been made exploring new antimicrobial drugs from natural sources including plant and animal products.

Approximately 20 million metric tones of fish by-products are discarded annually from the world fisheries. Fish by-products are rich in potentially valuable proteins, minerals, enzymes, pigments or flavours. Off the fish by products fish mucus is considered more valuable and has been reported that it contains antimicrobial proteins. In fish the epidermal mucus is considered a key component of innate immunity and plays a role in the prevention of colonization by parasites, bacteria and fungi[2-6]. The epidermal mucus, primarily produced by epidermal goblet or mucus cells, is composed mainly of water and gel-forming macromolecules including mucins and other glycoproteins[7,8]. The composition and rate of mucus secretion has been observed to change in response to microbial exposure or to environmental fluctuations such as hyperosmolarity and pH[9-11]. The mucus layer on the fish surface performs a number of inevitable functions including disease resistance, respiration, ionic and osmotic regulation, locomotion, repro-

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duction, communication, feeding and nest building[3,7,12]. It has been known that fish mucus contains a variety of biologically active compounds such as lysozyme, lectins, proteolytic enzymes, flavoenzymes, immunoglobulins, C-reactive protein, apolipoprotein A-I and antimicrobial peptides that are constitutively expressed to provide immediate protection to fish from potential pathogenic microbes and parasites[13-17]. An increased mortality rate has been reported in several fish species when removal of epidermal mucus and after challenging them with pathogenic microbe *Listonella anguillarum*[18,19]. The loss of epidermal mucus increased the rate of susceptibility to bacterial infection in carp (*Cyprinus carpio*)[5].

Antibacterial activity in fish mucus has been demonstrated in several fish species[20]; yet this activity seems to vary from species to species such as rockfish (*Sebastes schlegelii*)[17], rainbow trout (*Oncorhynchus mykiss*)[21] and tilapia (*Tilapia hornorum*)[22] and can be specific towards certain bacteria[23]. The exploration of the antimicrobial properties of epidermal mucus of Malaysian freshwater fish species is very limited and to date no studies have been made especially in snakehead fish, *Channa striatus*. Hence in the present study an attempt was made to find an antibacterial activity of the mucus extracts of snakehead fish *Channa striatus* against selected human and fish pathogens.

Materials and Methods

Fish Collection and Maintenance

Mucus sample was collected from snakehead fish, *Channa striatus* (body weight; 186.6 g ± 7.436 g) that were obtained from a local fish market in Sungai Petani, Kedah Darul Aman, Malaysia. Then the fish were stocked into the 500 L capacity circular plastic tanks. The fish acclimatized to laboratory conditions in a tap water and they were maintained for one week. During this period the fish were fed with commercial feed once a day at *ad libitum*. Every day 50 % of the water was changed. After one week of acclimatization the fish were used for mucus collection. Only healthy fish were chosen for mucus collection. Dead fish or fish with skin lesions were removed from the tanks.

Mucus Collection

Mucus was collected by a modified method of Subramanian et al[24]. Fish was starved for one day prior to mucus collection. On the day of mucus collection fish was washed and transferred into a sterile polyethylene bag for 10 to 20 minutes and moved front and back to slough off the fish mucus. Then, the fish was returned to recovery tanks. Fish mucus samples obtained from five fish was then pooled and stored in refrigerator at 4°C until further use. The pooled mucus sample was then divided into three parts, which were extracted separately with crude, acidic, and aqueous solvents.

Mucus Extraction and Protein Quantification

For crude extract, 50 ml of fish mucus was centrifuged at 5000 rpm for 10 minutes. The supernatant obtained was then stored in refrigerator at 4°C[25]. The aqueous extract of fish mucus was prepared using a method as described by Hellio et al[26]. Fifty ml of fish mucus was mixed with 50 ml of distilled water and homogenized using a polytron homogenizer. The mixture was then centrifuged at 30,000 g for 30 minutes at 4°C (Beckman coulter, Avanti J-26 XPI, Brea, CA, USA). Supernatant was then collected and filtered with Whatman no.1 filter paper. The filtrate was then collected and stored in refrigerator at 4°C. The acidic extract of fish mucus was prepared by using a modified method of Subramanian et al[24]. Thirty mL of the fish mucus was mixed with 30 mL of 3% acetic acid and placed in a boiling water bath for 5 minutes. The acid-mucus mixture was then cooled in ice and homogenized using polytron homogenizer. The mixture was then centrifuged at 18,000 g for 35 minutes at 4°C. Then, the supernatant was collected and purified using a syringe with 0.22 µm filter. Elutes were then collected and stored in refrigerator at 4°C. Protein quantification was determined based on Bradford protein assay[27] by using bovine serum albumin as standard.

Bacteria Culture Conditions

Antimicrobial activities of mucus extracts were tested against a range of human and fish pathogens including both Gram positive (*Bacillus subtilis*) and Gram negative bacterium (*Aeromonas hydrophila*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*). All the microbes were grown at 37°C in Luria-Bertani (LB) broth and maintained at 37°C in Luria-Bertani (LB)

agar except fish pathogen *Aeromonas hydrophila*. The fish pathogen was grown in Nutrient broth at 37°C.

Screening for Antimicrobial Activity

Preliminary screening for antimicrobial activity of all three mucus extracts prepared from *Channa striatus* was carried out against human pathogens (*Bacillus subtilis*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) and fish pathogen (*Aeromonas hydrophila*) using the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc susceptibility testing method[28]. Briefly, 20 µl of mucus extract was impregnated onto a disc. The disc with mucus extract was then transferred into the Mueller Hinton agar plate with bacterial culture. The optical density of culture was compared with 0.5 McFarland standard at 640 nm prior to plating. After the introduction of disc, the plate was then incubated at 37°C for 16 to 18 hours. Similar procedure was tested on the control by using solvent (distilled water, 3% acetic acid). The antimicrobial activity test was carried out in triplicate and the results were determined by observation of zone of inhibition. The results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs indicated the antimicrobial activity. All data on antimicrobial activity are the average of triplicates analyses.

Minimum Inhibitory Concentration (MIC)

Mucus extracts that showed antimicrobial activity was further subjected to test on minimum inhibitory concentration (MIC) which represents the lowest concentration of mucus extract that inhibited the growth of microorganism. The MIC test was carried out by using broth microdilution method as described by Subramanian et al[24] with slight modification. Mucus extract was serially two-fold diluted with 100 µl with Mueller-Hinton broth (HImedia, Mumbai, India) in order to determine the minimum concentration that can be used to inhibit the growth of microorganism. Fifty µl of overnight inoculum was then added into each tube containing different concentration of mucus extract and incubated at room temperature for 16 to 18 hours. Growth inhibition was observed by visual inspection of the turbidity of the mixture.

Statistical Analysis

One way analysis of variance and Duncan's multiple-range tests were employed to analyze data collected for zone of inhibition. Differences between means were considered significant when $P < 0.05$.

Results

In this study three different extraction methods was used to screen epidermal mucus for antimicrobial activities. Crude, acidic and aqueous extracts were prepared from the epidermal mucus of snakehead fish *Channa striatus*. The mucus was extracted with acidic solvent (acetic acid) in order to obtain a basic peptide/protein enriched extract of the mucus[29]. An aqueous extraction protocol was used to prepare an extract containing all the aqueous soluble components in the mucus, such as proteases, lysozyme and glycoproteins[30-32]. The results of the antibacterial activity of mucus extracts of *Channa striatus* are presented in Table I. These extracts were then

Table I. Screening of antimicrobial activity of the mucus extracts of snakehead fish *Channa striatus*.

Pathogens	Crude extract	Aqueous extract	Acidic extract
Human pathogens			
Bacillus subtilis	–	–	+ (7 mm)
Klebsiella pneumoniae	–	–	+ (11 mm)
Salmonella enteritidis	–	–	–
Proteus vulgaris	–	–	–
Pseudomonas aeruginosa	–	–	+ (10 mm)
Fish pathogen			
Aeromonas hydrophila	+ (8 mm)	+ (8 mm)	–

+ indicates antimicrobial activity; – indicates no antimicrobial activity, parenthesis values indicates the zone of inhibition. The values are the means of triplicates.

screened against five human pathogens *Bacillus subtilis, Klebsiella pneumoniae*, *Salmonella enteritidis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* and one fish pathogen *Aeromonas hydrophila* by using disc diffusion method. Antimicrobial activity was confirmed by the zone of inhibition. Among the three extracts, the crude and aqueous extracts showed a detectable level of bactericidal activity against the fish pathogen *Aeromonas hydrophila* but no activity against human pathogens. In contrast, the acidic mucus extract of *Channa striatus* showed antimicrobial activity against human pathogens *Bacillus subtilis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* but not the fish pathogen. The controls incubated with solvents and bacterial culture showed negative results, demonstrating that the solvents themselves did not account for the antimicrobial activity observed in fish mucus extracts. Maximum zone of inhibition for the acidic mucus extract was observed against human pathogen *Klebsiella pneumoniae* (11 mm in diameter), followed by *Pseudomonas aeruginosa* with a inhibition zone of 10 mm respectively. On the contrary, least inhibition (7 mm) was observed against *Bacillus subtilis*. The zone of inhibition for the crude and aqueous mucus extract against fish pathogen *Aeromonas hydrophila* was 8 mm respectively. The minimum inhibitory concentration of different mucus extracts are shown in Table II. The results revealed that a concentration of 0.2945 µg/ml in crude extract and 0.1455 µg/ml in aqueous extract was found to inhibit the growth of *Aeromonas hydrophila.* The minimum acidic mucus extract concentration of 0.066 µg/ml was found to inhibit the growth of human pathogens *Bacillus subtilis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The protein content of the three mucus extracts sample was shown in Table III. The highest amount of protein content (0.589 mg/ml) was observed in crude extract, followed by aqueous extract (0.291 mg/ml) and acidic extract (0.267 mg/ml) respectively.

Discussion

There is a wide range of pathogenic and non-pathogenic microorganisms that are in close association with fish in aquatic environment. Fish epidermal mucus plays a vital role in maintaining fish health providing a physical and biochemical barrier between the animal and the environment[8,33]. There are numerous studies on innate immune factors in fish epidermal mucus, including the role of proteases, antibacterial agents[26,34,35] and suggested that the epidermal fish mucus can inhibit the growth of some bacteria and therefore may have a potential source of novel antimicrobial components in it. Several studies have been carried out to explore the properties of fish mucus, whereas no information is available for the antimicrobial properties of snakehead fish, *Channa striatus* mucus.

In the present study, epidermal mucus of *Channa striatus* was collected and extracted in three different ways to obtain different components of the mucus. Protein quantification results revealed that *Channa striatus* contains a high amount of proteins that may be a potential antimicrobial source. The biochemical substances of mucus have been showed to differ depending on the ecological and physiological conditions such as salinity, pH, handling stress and stages of growth and maturity[36,37]. The variations in amount of mucus secretion between fish species had been observed to play a role in the susceptibility of the fish to infection. Previous studies have shown that fish mucus contains variety of enzymes including lysozymes, proteases, alka-

Table II. Minimum inhibitory concentration of the mucus extracts against human and fish pathogens.

Pathogens	Crude extract (µg/ml)	Aqueous extract (µg/ml)	Acidic extract (µg/ml)
Human pathogens			
Bacillus subtilis	–	–	0.066
Klebsiella pneumoniae	–	–	0.066
Pseudomonas aeruginosa	–	–	0.066
Fish pathogen			
Aeromonas hydrophila	0.2945	0.1455	

Table III. Protein content of different mucus extracts of snakehead fish *Channa striatus*.

Mucus sample extract	Protein content (mg/ml)
Crude	0.589
Aqueous	0.291
Acidic	0.267

line phosphatase and cathepsin B that play a significant role in innate immune system of fish[35]. Besides a histone H1-derived antimicrobial peptides, onchorhyncin II was also found in the skin secretions of rainbow trout[38].

Antimicrobial screening results showed that no detectable levels of antimicrobial activity observed in crude and aqueous mucus extract against human pathogens tested. Screening crude and aqueous mucus extracts inhibited the growth of fish pathogen *Aeromonas hydrophila*. This suggested that antimicrobial components might be present in the mucus. Earlier studies also have reported that, no microbial growth inhibition observed in aqueous fish mucus extracts of a wider range of fish species including Arctic char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), koi carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), haddock (*Melanogrammus aeglefinus*) and hagfish (*Myxine glutinosa*)[24]. Further, the antimicrobial activity of epidermal mucus extracted with acidic, organic and aqueous solvents varies remarkably within and among the fish species[24]. The absence of antimicrobial activity of the aqueous extracts in this study could be due to the presence of low levels of enzymes[24]. It has been reported that mucus enzymes may also influence the innate defense by activating the expression of genes that encode proteins such as antimicrobial peptides and complement proteins and could thereby impart antimicrobial activity through an indirect mechanism. For example, cathepsin D and matrix metalloprotease have been shown to be involved in the production of the antimicrobial peptide, parasin I, in the mucus of catfish *Parasilurus asotus*[39,40].

The acidic mucus extract of *Channa striatus* inhibited the growth of three human pathogens, *Bacillus subtilis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and not inhibited fish pathogen *Aeromonas hydropila*. The acidic mucus extracts of brook trout, haddock and hagfish showed bactericidal activity against a wide range of fish and human pathogens[24]. Similarly Hellio et al[26] reported that *Klebsiella pneumoniae* growth was inhibited by fish mucus extracts. Previous studies have shown a variety of antimicrobial proteins such as paradaxin and pleurocidin from fish mucus that was potentially involved in the protective function against invading pathogens[41,42].

The MIC test results showed that a minimum concentration of 0.2945 µg/ml of crude extract and 0.1455 µg/ml of aqueous extract was found to inhibit the growth of fish pathogen, *Aeromonas hydrophila*. The minimum concentration of 0.06675 µg/ml of acidic mucus extract was adequate to inhibit the growth of three human pathogens *Bacillus subtilis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. In the literature, some mucus extracts have been found to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* and the MIC values obtained were in the similar range as in the present study. Thus mucus fraction of eel, tench, trout, turbot, carp[43], winter flounder[42] and moses fish[41] inhibited the growth of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Escherichia coli, Aeromonas hydrophila and Staphylococcus aureus*.

In the this study the mucus isolated from *Channa striatus* shows an inhibiting effect on the selected microorganisms. The antibacterial activity of fish mucus may be due to the presence of antibacterial glycoproteins and able to kill bacteria by forming large pores in the target membrane[43]. Fish mucus is believed to play an important role in the prevention of colonization by parasites, bacteria and fungi and thus acts as a chemical defense barrier.

In conclusion, this research investigated the antimicrobial activities for snakehead head fish (*Channa striatus*) mucus extracts (crude, aqueous and acidic) against 5 human and 1 fish pathogens. From the results obtained, acidic extract inhibited the growth of three human pathogens, *Bacillus subtilis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Crude extract and aqueous extract inhibited the growth of fish pathogen *Aeromonas hydrophila* and none of the human pathogens. The present study also showed that mucus of *Channa striatus* can be a potential source of an antimicrobial activity for specific human and fish pathogens. In future, further investigations can be focused on other human and fish bacterial pathogens as well as fungal pathogens.

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