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Antimicrobial activity of different tissues of snakehead fish *Channa striatus* (Bloch)

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ABSTRACT

Objective: The aim of this study was to identify the presence of antimicrobial activity in different organs/tissues (gills, blood, skin, liver, intestine, kidney, tissue and ovary) extract of snakehead fish *Channa striatus*. **Methods:** A total of 48 fractions from the organs and tissue extracts were obtained by solid-phase extraction and the fractions were assayed for antimicrobial activity. The screening of antimicrobial activity for all the fractions were tested against 8 human pathogens including Gram positive (Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, *Bacillus cereus*) and Gram negative *bacteria* (*Salmonella enteritidis, Shigella flexneri, Acinetobacter baumanni, Escherichia coli, Klebsiella pneumoniae*) 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 results indicated that, among the 8 organs/tissues tested only blood and gills extract fractions (40 and 60 % ACN fraction) showed inhibition against *Escherichia coli* and 60 % ACN fraction of gill extract showed inhibition against *Salmonella enteritidis*. Protein profile analysis by SDS-PAGE showed that antimicrobial activity of the partially purified blood and gill tissue extracts might be due to low molecular weight peptides. **Conclusions:** The present study showed that, gill and blood extracts of *Channa striatus* can be a potential source of an antimicrobial protein for specific human pathogens.

1. Introduction

Tremendous improvements and advanced formulation in 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, annually 2 million people die due to diarrhoeal infections and diseases worldwide [2]. To combat this, several investigations have been focused to find out effective methods to prevent or cure diseases. Nowadays the development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for further attempts to search for new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents. Identifying a novel antimicrobial compounds which is inherent to specific organ and tissues of an organism could be the potential alternative to combat the drug resistant bacterial pathogens. In addition to the antimicrobial activity, such naturally occurring peptides may also play a critical role in the primary immune defense, as they prevent the colonization of opportunistic and pathogenic microorganisms. In this regard several studies have been made to explore the new antimicrobial drugs from natural sources including plant and animal origins. Antibacterial activity in fish mucus has been demonstrated in several fish species [3-7]. Very limited studies have been carried out in the screening of antimicrobial activities in different tissues of freshwater fish species [8]. Recently antibacterial activity of snakehead fish *Channa striatus* mucus has been reported and the exploration of the antimicrobial properties of different tissues/organs of C. striatus is not available [9]. Hence the present study was attempted to find the antibacterial activity of the various organs/tissues extracts of snakehead fish *Channa striatus* against selected human pathogens.

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2. Materials and Methods

2.1. Fish Collection

Ten healthy snakehead fish (*Channa striatus*) were obtained from a local fish supplier, Sungai Petani, Kedah Darul Aman, Malaysia and were maintained in circular cement tank (200 L capacity) at the aquaculture laboratory AIMST University. The fish were stocked at the density of 5 fish per tank at 29 ℃ and fed with small live trash fishes during the acclimation period.

2.2. Sample Collection

Eight different tissues and organs such as gills, skin, intestine, kidney, liver, blood, muscle and ovary were collected.

Each tissue from 10 fish were pooled for protein extraction and antimicrobial study. Before sampling experimental fish were anaesthetized with a sub lethal dose (100mg) of tricaine methanesulphonate (MS-222, Argent Chemical Laboratories, USA). Blood samples were collected from the caudal vein without anticoagulant using a 5ml sterilized plastic syringe, fitted with a 20 gauge needle. The operculum was removed to sample the gill filament. Following aseptic procedures, the internal organs and tissues skin, liver, intestine, kidney and ovary were collected. The muscle tissue samples were collected from the dorsal side of the body. The gut was separated and its contents were flushed out gently with PBS solution. The samples were pooled, weighed and immediately transferred to a bottle containing 0.5ml of protease inhibitor cocktail for general use (Sigma-Aldrich Corporation, USA). They were immediately frozen in liquid nitrogen and stored at -80℃ to inhibit the growth of bacteria and other microbes.

2.3. Sample Extraction

An organic solvent in acidic condition was used in the preparation of crude extracts [10]. Briefly, 5g of the pooled tissue or organ were mixed with 10 ml of solution containing 50% (v/v) ethanol, 3% (v/v) trifluoroacetic acid (TFA) and 2% (v/v) protease inhibitor cocktail. The acid tissue mixture was cooled and homogenized using a homogenizer following extraction by stirring for 60 min and then centrifuged at 18,000 g for 60 min at 4℃. The supernatant was collected, lyophilized and resuspended with 10 ml of 0.15%TFA and purified with reverse-phase Sep-Pak-Vac 5g 18 c cartridge. Prior to the addition of supernatant, the cartridge was activated with 10 ml of methanol and equilibrated with 10 ml of 0.15% TFA. After loading the supernatant, the flow through fraction was collected initially (F1) and second fraction (F2) was collected after the cartridge was washed with 10 ml of 0.15% TFA. The cartridge was then eluted with 10 ml of each of serial dilution of acetonitrile (ACN) in 0.15%TFA i.e. 20%, 40%, 60%, 100% ACN (v/v) and each fraction (F3, F4, F5 and F6) was collected in order to prepare the small aqueous soluble molecules and the more polar and less polar sub-fractions. The fractions obtained (F1-F6) were lyophilized and resuspended in 20Mm HEPES pH7.0 to give a concentration of approximately 1 mg dry substance/ ml. The pH was adjusted in the range of 6-8 with 0.1N NaoH and the preliminary test for antimicrobial activity was carried out against various strains of bacteria.

2.4. Screening for Antimicrobial Activity

The following Gram positive bacteria (Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, *Bacillus cereus*) and Gram negative bacteria (*Salmonella enteritidis, Shigella flexneri, Acinetobacter baumanni, Escherichia coli Klebsiella pneumoniae*) were used to test the antimicrobial activity. Preliminary screening for antimicrobial activity of all fractions from different tissues and organs of *Channa striatus* was carried out against all the above human pathogens using the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc susceptibility testing method [11]. Briefly, 20 μl of eluted fractions was impregnated onto a disc. The disc with each fraction was then transferred into the agar plate with bacterial culture. The optical density of culture was compared with 0.5 McFarland standards at 625 nm prior to plating. Antibiotics such as chloramphenicol, ciprofloxacin, and amikacin were used as positive control for all the bacterial strains. After the introduction of disc, the plate was then incubated at 35°C for 16 to 18 hours. The antimicrobial activity test was carried out in triplicate and the results were recorded by measuring the zone of inhibition (mm) surrounding the disc. Clear inhibition zones around the discs indicated the antimicrobial activity.

2.5. SDS-PAGE

Protein profiles of tissue/ organ samples showed antibacterial activity were determined by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) running in Tris-Tricine buffer system with a 15% separating gel and 5% stacking gel. Ten microliters of sample extract and 10 ml of protein loading buffer [4% (w/v) SDS, 12% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 0.61% (w/v) Tris, 2% (w/v) mercaptoethanol, pH 6.8] were mixed and boiled at 100 ℃ for 5 min before loading into each well of the gel. The gel electrophoresis was run approximately for 2 h at 100 V (Bio-Rad, USA). The gels were stained using Coomassie brilliant blue [0.25% (w/v) Coomassie blue in 25% (v/v) methanol and 10% (v/v) glacial acetic acid] for 15 min and de-stained overnight with 7.5% (v/v) glacial acetic acid in 50% (v/v) methanol and visualized. The masses of the proteins were determined by comparison with protein standard.

3. Results

In the present study, the extracts were made from 8 different organs and tissues (gills, blood, skin, liver, intestine, kidney, tissue and ovary) from snakehead fish *Channa striatus* and they were partially purified by solid phase extraction and eluted into 6 fractions. A total of 48 fraction samples were obtained and each fraction was assayed for antibacterial activity against various strains of Gram-positive and Gram-negative human bacterial pathogens. The results indicated that, of the 48 fractions tested from 8 different organs/tissues of snakehead

fish only blood and gills extracts fractions (40% and 60 % ACN fraction) showed inhibition against *Escherichia coli* and 60 % ACN fraction of gill extract against *Salmonella enteritidis* (Table 1). Interestingly, the antibiotic sensitivity study revealed that, despite the antibiotic resistance of E.coli against tested antibiotics such as chloramphenicol and ciprofloxacin, the bacteria was found to be susceptible to the fractions of blood and gill extracts. Similarly, *S. enteritidis* which showed resistance to ciprofloxacin and amikacin was also found to be susceptible to the fractions of blood extract. The other fractions from skin, liver, intestine, kidney, tissue and ovary extracts did not inhibit the growth of the bacteria.

3.1. SDS-PAGE

The SDS-PAGE protein profiles of the blood and gill extract fractions is shown in Fig 1. The protein profiles showed a broad range of peptides and proteins, with molecular weights ranging from less than 10 kDa to over 130 kDa. The small proteins with molecular weights less than 17 kDa were abundant in the 40% and 60% ACN fraction from blood and gill extracts. Few protein bands were observed in gill extract, flow-through and 0.15% TFA fraction and 20% ACN fraction.

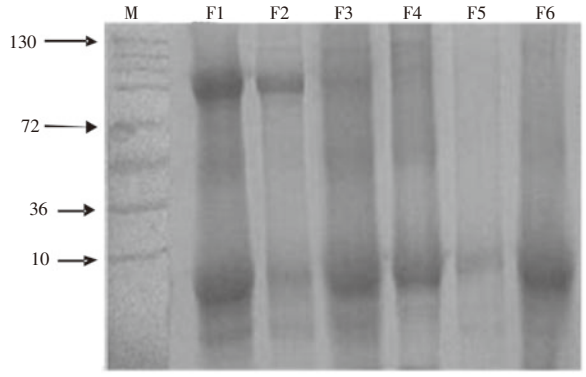

Figure 1. SDS-PAGE profile of different fractions of blood extracts of snakehead fish *Channa striatus*. M: Molecular weight markers. Lane 1- 6: F1- Flow-through fraction; F2- 0.15% TFA fraction; F3- 20% ACN fraction , F4- 40% ACN fraction, F5- 60% ACN fraction; F6- 100% ACN fraction.

Table 1

Screening of antimicrobial activity of the blood and gill extracts of snakehead fish *Channa striatus* against *Escherichia coli* (EC), *Klebsiella pneumoniae(KP), Salmonella enteritidis(SE), Shigella flexneri (SF), Acinetobacter baumanni (AB), Methicillin-resistant Staphylococcus aureus (MRSA), Staphylococcus aureus (SA)* and *Bacillus cereus (BC)*.Values are the mean of inhibition zone (mm) of 3 replicates.

Fractions	Zone of inhibition with blood extract fraction against human pathogens							
	EC	*KP*	*SE*	*SF*	*AB*	*MRSA*	*SA*	*BC*
F1	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	-	-
F3	-	-	-	-	-	-	-	-
F4	9	-	-	-	-	-	-	-
F5	12	-	8	-	-	-	-	-
F6	-	-	-	-	-	-	-	-
	Zone of inhibition with gill extract fraction against human pathogens							
F1	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	-	-
F3	-	-	-	-	-	-	-	-
F4	8	-	-	-	-	-	-	-
F5	11.2	-	-	-	-	-	-	-
F6	-	-	-	-	-	-	-	-
Chloramphenicol	-	-	27	-	25	12	-	12
Ciprofloxacin	-	-	-	32	-	-	34	-
Amikacin	18	16	-		-	-	-	-

F1- Flow-through fraction; F2- 0.15% TFA fraction; F3- 20% ACN fraction , F4- 40% ACN fraction, F5- 60% ACN fraction; F6- 100% ACN fraction.

4. Discussion

Recently there is a growing interest in exploring natural antimicrobial drugs from terrestrial and aquatic living organisms. Approximately 20 million metric tones of fish by-products are discarded annually from the world fisheries [13]. Fish by-products are rich in potentially valuable proteins, minerals, enzymes, pigments and also contain antimicrobial agents. Among the fish by products fish mucus, gills and blood is considered more valuable and has been reported that it contains several antimicrobial proteins [12, 13].

In the present study we elucidated the antibacterial activity of 48 fractions from 8 different organs/tissues of snakehead fish. There was no activity in the flow-through fractions of most of the tissues and organs extracted, except for the blood and gill extract. Antibacterial activity was found only in 40% and 60% ACN fractions of blood extract against *Escherichia coli* and 60% ACN fraction of gill extract against *Salmonella enteritidis*. This indicates that the hydrophilic and hydrophobic substances are not the potent antibacterial compounds in the blood and gill extracts. A similar result was reported in 50% and 70% of ACN fraction from Atlantic cod (*Gadus morhua*) tissue and organ extracts8. No inhibiting activity was observed in the flow through fractions of rest of the tissues and organ extracts in the present study. In general, no antimicrobial activity has been reported in the epidermis and epidermal aqueous mucus extract or in the dichloromethane and aqueous phase of organic mucus extract of various fishes [9, 12, 13]. The antibacterial

activity detected in 40% ACN fraction isolated from blood and gills extract could be attributed to water soluble basic proteins such as trypsin, chymotrypsin and serine protease. It has been reported that, the gill tissue act as the first barrier to combat infection by trapping and sloughing off pathogens, since they contain numerous mucous cells, mast cells and leukocytes [14,15]. In addition, the mucosa related tissues contain several components for fighting the invading organisms, for e.g. lectins, pentraxins, lysozyme, complement proteins and IgM [15-18]. Proteins with antimicrobail properties have been isolated from gills of different fish species chrysophsin-1, 2 and 3 from red sea bream, Chrysophrys major[19]. Blood contains numerous lymphoid cells, cells in circulation that are responsible for phagocytosis and production of antibodies, and other immunocompetent molecules [14]. Antibacterial agents are normally present in the serum of fish [20, 21]. The antibacterial proteinaceous factors have also been isolated from erythrocytes of rainbow trout [10] and from the peripheral blood leukocyte of channel catfish [22]. The protein profiles of blood and gill extracts fractions showed that, small proteins with molecular weights less than 10 kDa were visible in the 40% and 60% ACN fraction. These results confirm the presence of antibacterial compounds in blood and gills. SDS-PAGE analysis of soluble proteins eluted with 0.15% TFA fraction, 20% and 40% ACN fraction showed profiles with large molecular weight (>72 kDa) in blood and gill extract. The study also observed that, among the bacterial strains, *E. coli* exhibited resistance against chloramphenicol and ciprofloxacin; and *S. enteritidis* showed resistance against ciprofloxacin and amikacin. Interestingly the fraction of blood and gill extract inhibited the antibiotic resistant bacteria. In summary, this study demonstrates that only blood and gill extract fractions exhibited inhibitory activity against *Escherichia coli* and *Salmonella enteritidis*. The gill and blood extracts of *Channa striatus* can be a potential source of an antimicrobial protein for specific human pathogen. Further study is needed to purify the antimicrobial protein from blood and gill extract fractions of snakehead fish, *C. striatus*.

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Conflict of interest statement

We declare that we have no conflict of interest.

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