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Antimicrobial and Hemolytic Activity of the Fish Collagen Extracted from Freshwater Snakehead Fish *Channa striatus*

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Abstract
Collagen is the most predominant, abundant and major protein of connective tissue present in the animal body. The collagens are widely used in many pharmaceutical industries, food, healthcare, cosmetics and scaffold tissue engineering. In the present study effort has been made to identify the antimicrobial and hemolytic activity of the collagen extracted from the freshwater snakehead fish *Channa stiriatus.* Extracted fish collagen were tested against the four pathogenic bacteria viz., *Escherichia coli*, *Staphylococcus aureus, Bacillus Subtilis* and *Klebsiella Pneumoniae,* four pathogenic fungi namely *Aspergillus flavus, Aspergillus nidulans, Candida albicans,* and *Fusarium moniliforme*. The hemolytic activity was tested on chicken and goat blood erythrocytes. The collagen extracted from freshwater fish shows a strong antibacterial and hemolytic activity.

Keywords
Collagen, snakehead fish, Channa striatus, hemolytic, antibacterial and antifungal.

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INTRODUCTION
Collagen is major extracellular protein of matrix plays a major role in maintaining physiological functions with diverse biomedical applications which include tissue engineering, food processing industry, manufacturing of cosmetics, biofilms and mostly in pharmaceutical industries. The bovine spongiform encephalopathy and transmissible spongiform encephalopathy initiate the researchers for the isolation of collagen from the alternative sources rather than from the cattle. One of the alternative sources is the invertebrates of which fishes are used for extracting the collagen. The fish collagens are having low melting temperature, lower gelling, fibrillar and non-fibrillar protein substances [1]. The collagen extracted from the fishes are heat sensitive due to labile cross links and reduced level of hydroxyproline [2]. The inertness structural ability and biocompatibility of collagen possess a promising anticancer activity and ophthalmic drug delivery system [3, 4].
Among the collagen alternatives, fishes are the best source because of its high availability, less risk in disease transmission, religious barrier and rich protein content. Substantial amount of collagen could be obtained from fish which provide an alternative source to bovine collagen in food, cosmetics and pharmaceutical applications.

Collagens are easily extracted and purified from the skin especially from the freshwater fish. Numerous attempts have been recently made to study the use of collagen extracted from the fishes. The application of jellyfish collagen containing telopeptides enhances the production of IgM in the human hybridoma cell line.

A number of alternative source for collagen have been investigated in recent times including freshwater fishes, marine fishes, chicken skin, jellyfish, octopus, starfish and squid. However, the application of collagen extracted from these sources will be realized upon immunological characteristics, biocompatibility and less side effects. The presence of collagen in organs and most tissues, its biochemical structure and biomedical uses initiate the scientists in finding collagen sources in different animals especially on fishes.

C. striatus, or snakehead murrel, is an obligate air-breathing freshwater fish which inhabits all types of water bodies from small ditches to rice fields, rivers and lakes across tropical and subtropical Asian countries from Pakistan and India to Southeast Asia and Southern China [5, 6]. *C. striatus* is commonly consumed as a food fish. In India, freshwater fish consumption provides an important source of protein constituting up to 70% of total protein requirements [7] and is also recognized as a source of omega-3 fatty acids [8]. *C. striatus* features prominently in the local diet among the India and Malaysia,[9] and tribal communities in East Malaysia [10].

C. striatus is also highly valued for its medicinal properties. The popularity of *C. striatus* as a therapeutic agent is related to folk belief in its efficacy in treating wounds, relieving pain and boosting energy in the sick and elderly. Mothers recuperating from normal or Caesarean delivery [11] and patients recovering from surgical operations are routinely and customarily advised to eat meals containing *C. striatus* [12]. Current study deals with the antimicrobial and hemolytic activity of the collagen isolated from the freshwater snake head fish *Channa striatus*.

2. MATERIALS AND METHODS

2.1. Collection of fish

The healthy *C. striatus* were collected from Sirkali fish market, Nagapatinam District, Tamilnadu, India of an average weight 300 ± 5.67g. The fish were kept in large aerated concrete tank containing potable tap water (pH 7.5 ± 0.5). The tank was treated with disinfectant sodium hypochloride, with the concentration of 200 ppm for 1 hrs and washed three times with fresh tap water prior to the introduction of the fish in the water.

2.2. Preparation and extraction of Acid-Solubilized Collagen

The Acid-Solubilized collagen was extracted by following the method of Hema *et al* [2] with slight modification. All the extraction procedures were carried out at 4 ºC. The fish skin was minced and mixed with 30 volumes of 0.1N sodium hydroxide and kept stirred for 24 hours over a magnetic stirrer to remove non collagenous protein. The treated mass was strained through a coarse sieve. The process was repeated twice, and the residue was washed twice with 30 volumes of chilled distilled water.

The residue was homogenized in a Polytron homogenizer with 30 volumes of 0.5M acetic acid for one minute and the same was stirred over a magnetic stirrer for 24 hours. The supernatant after centrifugation (3000 rpm, 20 min) was collected. The residue was once again extracted with acid as anove and the combined supernatant was taken as acid soluble collagen.

Crystalline sodium chloride was added to supernatant to the level of 10% and stirred for 24 hours to precipitate the collagen. The precipitate was suspended in Tris-glycine buffer (50 mM containing 0.2M NaCl, pH 7.4) and dialyzed against the same buffer for 24 hours and then centrifuged. The collagen obtained was spray dried to get fine powder.

2.3. Microbial strains used

Antimicrobial activity of fish collagen was determined against four bacterial strains viz, *Escherichia coli*, *Staphylococcus aureus, Bacillus Subtilis* and *Klebsiella Pneumoniae,* four pathogenic fungi namely *Aspergillus flavus, Aspergillus nidulans, Candida albicans,* and *Fusarium moniliforme*. These pathogenic strains were obtained from the division of microbiology, Rajah Muthiah Medical College, Annamalai University and cultured as stock for presuming microbial activity. From the stock 18 hours old cultures for bacteria and 2 days old cultures for fungi were used for swabbing.

2.4. Antimicrobial activity

The spectrum of antimicrobial activity was studied using the above-mentioned bacteria and fungi which are pathogenic to human and fish. In the present study a standard positive control is used for both bacteria and fungi (erythromycin for bacteria and fluconazole for pathogenic fungi). *In vitro* antibacterial assay was carried out by the disc diffusion technique [13]. Whatmann No. 1 filter paper discs with 6 mm diameter were impregnated

with different concentration (25µl, 50µl, 75µl and 100µl) of test sample fish collagen and positive control contained a standard antibiotic. Sterile disc is used as a negative control. The impregnated discs along with control were kept on agar plates, seeded with test bacterial cultures (37°C for 24 hours) and fungal culture separately. At room temperature the bacterial plates were incubated for 24 hrs and the fungal plates at 30°C for 48 hours were incubated separately to find out the antifungal activity. The activities of mucus against microbes were expressed in terms of diameter of zone of inhibition and measured in millimeter using cm scales and recorded. In each strain triplicate were maintained, the average was taken and tabulated.

Haemolytic activity

The Acid solubilized collagen of *C. striatus* were assayed to identify the heamolytic activity on chicken and goat blood erythrocytes followed by the method of Paniprasad and Venkateshvaran [14].

4.6.1. Preparation of erythrocytes suspension

The chicken and goat blood were obtained from the slaughterhouse Annamalainagar and was added with 5% EDTA (Ethylene Diamine Tetra-acetic Acid) solution as an anticoagulant of blood. The samples were centrifuged thrice at 5000 rpm for 5 minutes; 1 % erythrocytes suspension was prepared by adding 99ml normal saline to 1ml of packaged erythrocytes.

4.6.2. In vitro Haemolytic assay

The in-vitro haemolytic assay was performed in 96-well 'V' bottom microtitre plates. Serial two-fold dilutions of the fish collagen were made in 100µl of phosphate buffer solution. Then 100 µl of 1% erythrocytes was added to all the wells. For positive control, 100 µl of distilled water was added respectively to the 1% red blood cell (RBC) suspension and PBS solution was used as negative control. The plates were gently shaken and allowed to stand for two hrs at room temperature. The presence of uniform red colour suspension in wells was considered to be positive haemolysis and a button formation in the bottom of the wells constituted a lack of haemolysis. The reciprocal of the highest dilution of the extract shows the haemolytic pattern was taken as one haemolytic unit (HU).

RESULTS

Antimicrobial assay

The potential of antimicrobial activity of the epidermal collagen of freshwater snakehead fish *C. striatus* were tested against four, bacterial strains and four pathogenic filamentous fungi at different concentrations (25 µl, 50 µl, 75 µl and 100 µl). The activity was measured in terms of zone of inhibition and expressed in millimeter (mm).

5.6.1. Antibacterial activity of the skin collagen

The inhibitory effects of the epidermal collagen of freshwater snakehead fish *C. striatus* against five pathogenic bacterial strains (*Staphylococcus aureus, Escherichia coli, klebsiella Pneumoniae and Bacillus substillis*) are given in Table (1). The fish collagen shows a significant effect by increasing its concentration. In all the bacterial strains tested maximum activity was observed in 100 µl of fish collagen. The magnitude of zone of inhibition was *Escherichia coli > klebsiella Pneumoniae > Bacillus substillis > Staphylococcus aureus.* The maximum zone of inhibition was observed against *Escherichia coli* which are about 20.89 ± 1.15 mm in diameter (Fig-a). This was followed by *klebsiella Pneumoniae* (18.00 ± 0.15 mm in diameter) (Fig-b), *Staphylococcus aureus* (17.20 ± 0.85 mm in diameter) (Fig-c), and *Bacillus substillis* was 12.20± 1.15 mm in diameter (Fig-d), respectively. The zone of inhibition of collagen against the selected bacteria is given in Plate-1.

The collagen extracted from the fresh water snakehead fish *C. striatus* shows more resistance to the *Escherichia coli* (20.89 ± 1.15 mm) and least resistance to *Bacillus substillis* (16.20 ± 1.15 mm).

The fish collagen showed a significant activity with increasing concentration in all the bacterial strains tested. The zone of inhibition values of collagen of *C. striatus* and positive control (Streptomycin) were similar and the graphical representation is shown in Graph-1.

Table-1. Antibacterial activity of fish collagen

Sl.No	Bacterial pathogens	Zone of inhibition in mm				
		Fish collagen at different concentration				Positive control
		25 µl	50 µl	75 µl	100 µl	
1.	*Escherichia coli*	10 ± 0.15	13 ± 0.75	17 ± 0.95	20 ± 1.15	25 ± 0.50
2.	*Klebsiella Pneumoniae*	7 ± 1.13	10 ± 0.65	15 ± 1.25	18 ± 0.95	22 ± 1.05
3.	*Bacillus Subtilis*	3 ± 0.75	7 ± 1.05	13 ± 1.50	17 ± 0.85	19 ± 0.95
4.	*Staphylococcus aureus*	7 ± 1.05	10 ± 0.85	12 ± 2.05	16 ± 1.15	21 ± 0.65

❖ **Data are given in Mean ± Standard deviation of six observations**

Graph 1. Antibacterial activity of fish collagen

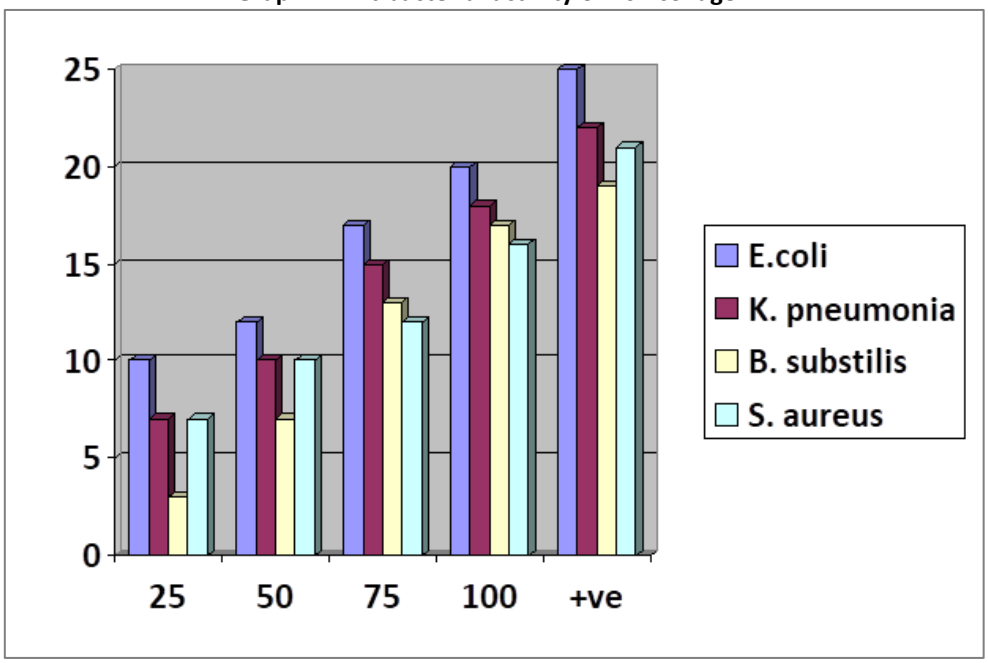

Plate-1. Antibacterial activity of fish collagen

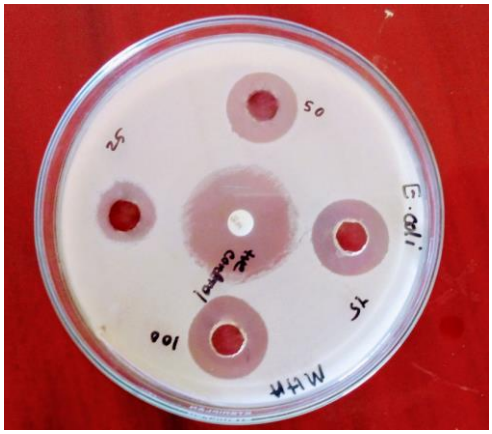

Fig a *Escherichia coli*

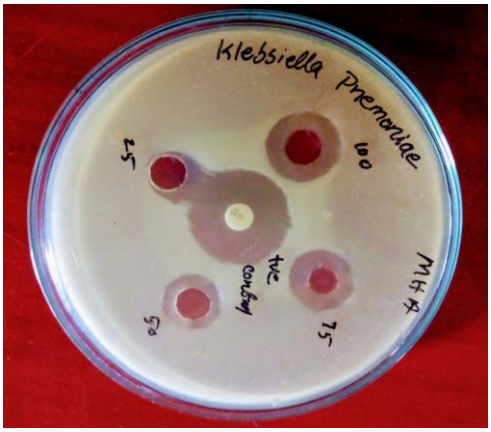

Fig b *Klebsiella pneumoniae*

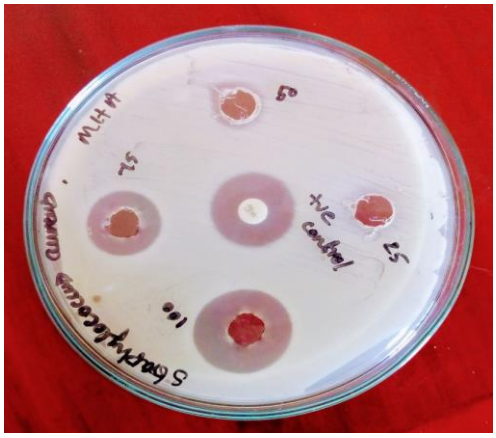

Fig c *Staphylococcus aureus*

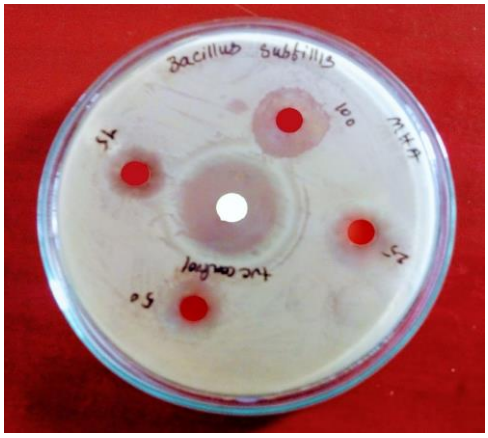

Fig d *Bacillus subtilis*

5.6.2. Antifungal activity of fish collagen

The antifungal activity of the collagen of freshwater snakehead fish *C. striatus* were investigated against the five filamentous fungal strains (*Aspergillus flavus, Aspergillus nidulans, Candida albicans,* and *Fusarium moniliforme*) and results are tabulated in Table-2. The fish collagen shows a significant effect by increasing its concentration. In all the fungal strains tested, maximum activity was observed in 100 µl of fish collagen. The magnitude of zone of inhibition was *Candida albicans > Fusarium moniliforme > Aspergillus flavus > Aspergillus Nidulans.*

The maximum zone of inhibition was observed against *Candida albicans* which is about 8.60 ± 1.85 mm in diameter (Fig-). This was followed by, *Fusarium moniliforme*is (7.20 ± 0.20 mm in diameter) (Fig-), *Aspergillus flavus* (6.60 ± 1.85 mm in diameter) (Fig-) and *Aspergillus nidulans* is about 4.80 ± 1.16 mm in diameter (Fig-f). The zone of inhibition of the fish collagen against tested fungi is given in Plate-2. The collagen collected from the freshwater snakehead fish, *C. striatus* shows more resistance to the *Candida albicans* (8.60 ± 1.85 mm) and least resistance to *Aspergillus nidulans* (4.80 ± 1.16 mm).

The collagen of snakehead fish showed a significant activity in all the concentration tested with regard to the filamentous fungal strains. The zone of inhibition values of collagen against fungal growth was relatively similar to the positive control and the graphical representation is shown in Graph-2.

Table-2. Antifungal activity of fish collagen

Sl no.	Fungal pathogens	Fish collagen at different concentration				Positive control
		25 µl	50 µl	75 µl	100 µl	
1.	*Aspergillus nidulans*	-	2.10 ± 1.16	3.20 ± 0.16	4.80 ± 1.16	7.80 ± 0.16
2.	*Aspergillus flavus*	-	3.05 ± 1.15	4.10 ± 0.95	6.60 ± 1.85	8.69 ± 0.85
3.	*Candida albicans*	-	2.90 ± 1.02	6.70 ± 1.35	8.60 ± 1.12	9.60 ± 0.50
4.	*Fusarium moniliforme*	-	3.15 ± 0.95	5.25 ± 1.20	7.20 ± 0.20	10.60 ± 1.85

❖ Data are given in Mean ± Standard deviation of six observations.

Graph 2. Antifungal activity of fish collagen

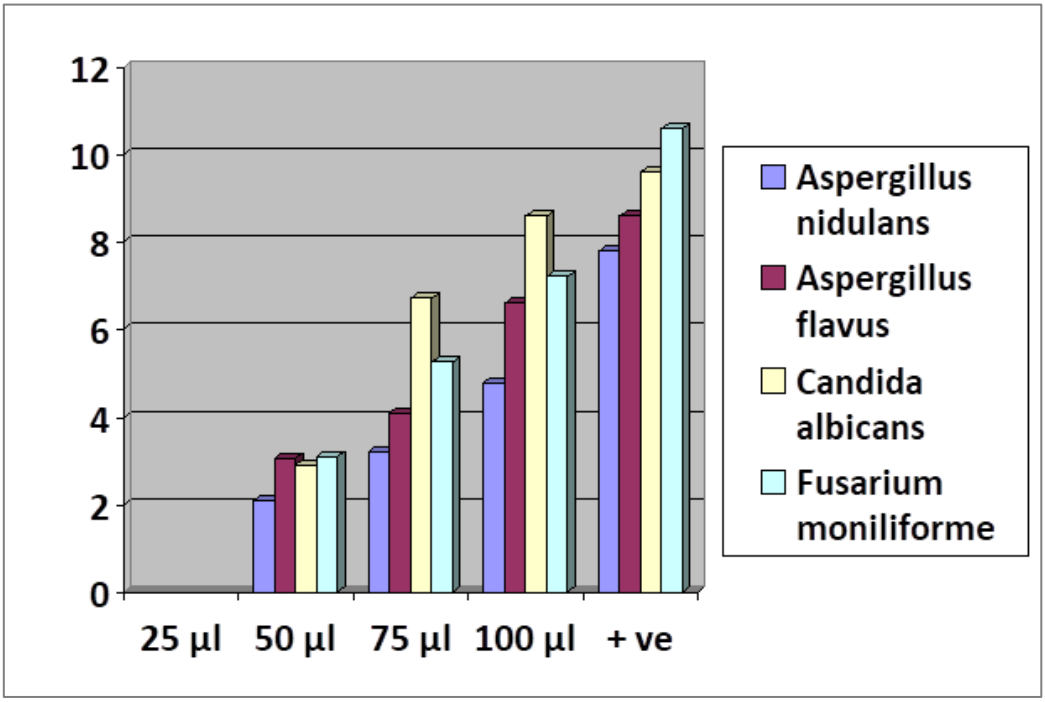

Plate-2. Antifungal activity of fish collagen

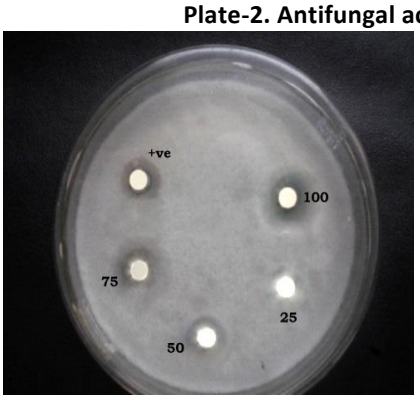

Fig-f. *Aspergillus flavus*

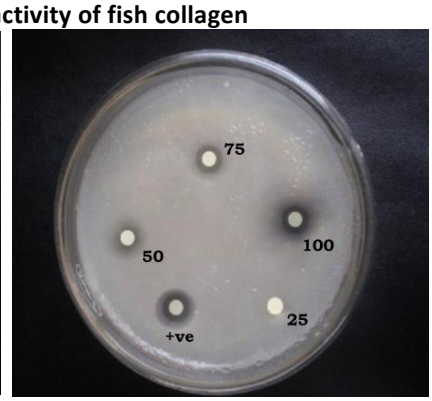

Fig-g. *Aspergillus nidulans*

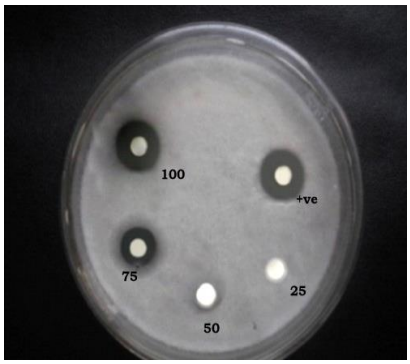

Fig-h. *Candida albicans*

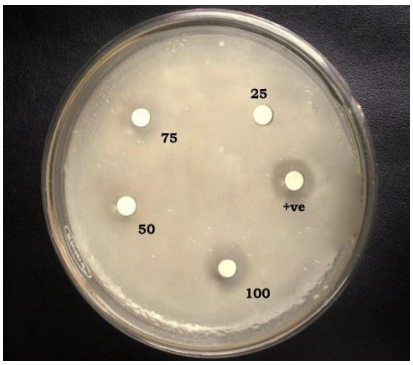

Fig i. *Fusarium moniliforme*

5.7. Haemolytic assay of fish collagen

The extracted epidermal collagen of *C. striatus* were tested for haemolytic activity on chicken and goat blood erythrocytes and its results were tabulated in Table. The collagen extracted from *C. striatus* showed good haemolytic activity against both animal (chicken and goat) blood erythrocytes (Fig-). The maximum Haemolytic Unit (HU) was recorded in chicken blood (8HU) and minimum was in goat blood (4HU). The specific haemolytic activity of fish collagen showed 0.0301 mg/ml in chicken and 0.0148 mg/ml in goat blood erythrocytes. The haemolytic unit of chicken blood was higher than the goat blood.

C- Chicken blood; G- Goat blood

Figure-4. Haemolytic activity of fish collagen

Table-4. Haemolytic activity of fish collagen

Sl. No	Blood samples	Toal protein µg/ml	Haemolysis	Haemolytic unit (HU)	Specific haemolytic unit µg/ml
1	Chicken blood	252± 0.56	3	8	0.0301
2	Goat blood	254± 0.56	2	4	0.0148

DISCUSSION

Fish are of great economic importance in aquaculture throughout the world. Intensive culture, the recent techniques for fish culture has increased the production but accompanied with the outbreak of many infectious diseases. Fish have evolved to thrive in an aqueous environment rich in microbial flora and are persumed to use their innate invasion. The main site of action of the majority of microbes is believed to be the plasma membrane, where they cause pore formation or membrane lysis [15]. Virtually all fishes are covered with integumental mucus that is involved in many aspects of their biology ranging from disease resistance to rearing of young ones to shelter and locomotion. It was reported that epithelial tissue produces antimicrobial molecules which serve as the first line of host's defense against microbial invasion in a variety of vertebrates including humans [16]

Collagens are easily extracted and purified from fish skin and bones with a maximum yield. Collagens are likely to present more in the epithelial tissue of fish. Collagen substrates are more to affect the growth of the infectious cells and modulate the various aspects of cell behavior, such as cell adhesion, proliferation and differentiation [17-19]. The collagen extracted from the freshwater fish *C. striatus* is composed of type I collagen fibrils which acts readily on the tested microbes. The fibrous collagen are generally present in the tissue in the form of covalent cross-linking between the individual proten subunits [20]. Goblet cells or mucous cells are important functional constituents of fish epithelia and are almost universally present in the skin with few exceptions. Fish also contain cellular interferons which possess anti-viral proteins, enzymes – inhibitors (α-macroglobulin and other α_2-globulins) that inhibit the extra cellular proteases secreted by pathogens. Number of relatively specific lytic molecules, like hydrolase enzymes (Lysozyme, Chinase and Chitobiase) are present in the mucus which acts on fungi and bacteria. Other than that, fish also contain lectins that possessed antifungal and antibacterial activities [21].

Fish tissues and body fluids contain naturally occurring proteins or glycoproteins of non-immunoglobulin nature (e.g. transferrins, caeruloplasmin and metallothionein) that react with a diverse array of environmental antigens and may concern an undefined degree of natural immunity to fish. Those compounds exhibit microbial growth inhibitory activity via simple metal ions chelating mechanism, which deprive microbes of essential inorganic ion sources [22]. Amphipathic α-helical peptides, such as dermaseptin, ceratotoxin and Magainin bind with anionic phsopholipid-rich membranes and dissolve them like detergents [23]. These peptides are known to exert action by binding to the surface of the microbial membranes and cause lysis of the intracellular contents [24]. These findings support our results that the antimicrobial activity of the collagen extracted from freshwater snakehead fish *C. striatus* may be due to the presence of the above said substances. The antimicrobial substance present in the mucus may function either in the cytoplasm against intracellular pathogens or extracellularly through release to mucosal surfaces after infection-induced cell lysis or apoptosis. A few antimicrobial agents structurally identified in collagen of bony fishes are proteins. It has been proposed that these compounds bind to and essentially dissolve cellular membrane [25-27]. Several studies support our findings that the freshwater snakehead fish *C. striatus* possess strong antimicrobial activity [28-30]

Haemolytic activity has been observed in many of the tissue products of aquatic organisms. The haemolytic activity of monomeric and dimeric cynthacurin compound isolated from the ascidian of sackcho, southkorea showed 21% of lysis against human red blood cells, an equal concentration of cynthacurin lacked haemolytic activity against other mammalian red blood cells [18]. Fishes exhibit a good source of substances having haemolytic activity. Hemolysis of human and sheep red blood cells have been studied by Al-Hassan *et al.* [31] They stated that the specific activity of the cat fish epidermal factor is 20.6 units mg^{-1}, a level somewhat lower than those of most protein haemolytic factor. In the present study the collagen extracted from the freshwater snakehead fish is having more amount of protein which shows good haemolytic activity. Most fish possess haemolytic activity and many have also been demonstrated to be cytolytic to other cell types, particularly muscle cells. Furthermore, this haemolytic activity is the result of pore-formation in cell membranes. Pore forming activity seems to be a common trait among many fishes [32-33].

Thus, the collagen extracted from the freshwater snakehead fish *C. striatus* shows a broad spectrum of antimicrobial and hemolytic activity can be subjected to further evaluation to analysis the chemical composition and to reveal the mode of action against microbes.

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