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Full Length Research Paper

Antimicrobial properties of skin mucus from four freshwater cultivable Fishes (*Catla catla, Hypophthalmichthys molitrix, Labeo rohita and Ctenopharyngodon idella)*

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The fishes are living in the medium rich in pathogenic microbes. The mucus secreted by the skin of fish showed more antimicrobial properties. The mucus collected from the two exotic fishes and two indigenous fishes were tested against the five pathogenic bacteria (*Klebsiella pneumonia, Vibrio cholerae, Salmonella typhi, Escherichia coli* and *Pseudomonas aeruginosa*) and five pathogenic fungi namely (*Mucor globosus, Rhizopus arrhizus, Candida albicans, Aspergillus flavus* and *Aspergillus niger*). The fishes are living in media rich in pathogenic microbes which secrete substances against them. The mucus secreted by the skin of fish showed more antimicrobial properties. More antibacterial and antifungal activity were observed in an indigenous fishes (*Catla catla and Labeo rohita*) than exotic fishes (*Hypophthalmichthys molitrix* and *Ctenopharyngodon idella*).

Key words: Antibacterial, antifungal, fish mucus, exotic, indigenous fish.

INTRODUCTION

Chemicals from nature have been a part of human civilization ever since our early ancestor's began exploiting natural compounds to improve and enrich their own lives (Agosta, 1996). A major part of these chemicals come from animals. Indeed, animals are therapeutic arsenals that have been playing significant roles in the healing processes, magic rituals, and religious practices of peoples (Costa and Marques, 2000). All living organisms including fish coexist with a wide range of pathogenic and non- pathogenic microorganisms and therefore, posses complex defense mechanisms which contribute to their survival. One mechanism is the innate immune system that combats pathogens from the moment of their first contact (kimbrell and Beutler, 2001). The specific immunity including antibody and specific cell-mediated responses are significantly less diverse than those of higher (Ellis, 1974; Manning, 1998).

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, which overcome the problems of resistance and side effects. Action must be taken to reduce this problem such as controlling the use of antibiotics, carrying out research to investigate drugs from natural sources. Drugs that can either inhibit the growth of pathogen or kill them and have no or least toxicity to the host cell are considered for developing new antimicrobial drugs. It is well known that the global trade in animal based medicinal products accounts for billions of dollars per year (Kunin and Lawton, 1996). Unlike conventional antibiotics, which are synthesized enzymatically by microorganisms, are encoded by a distinct gene (AMP) and made from an mRNA template. The continuous use of antibiotics has resulted in multi resistant bacterial strains all over the world (Mainous and Pomeroy, 2001). Consequently, there is an urgent need to search for alternatives

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to synthetic antibiotics. In spite of modern improvements in chemotherapeutic techniques, infectious diseases are still an increasingly important public health issue (WHO, 2002). It has been estimated that in 2000, at least two million people died from diarrhoeal disease worldwide (WHO, 2002). Still there is a need for new methods of reducing or eliminating pathogens, possibly in combination with existing methods (Leistner, 1978). In the aquatic environment, fish are in constant interaction with a wide range of pathogenic and non-pathogenic microorganisms (Subramanian et al., 2007).

Fish live in a challenging environment facing so many problems. The microbes play a major role in affecting the fish health. They escape from such an environment by producing some substances on the dermal layer (Mucus). The epidermal mucus produced primarily by epidermal goblet or mucus cells are composed mainly of water and gel forming macromolecules including mucins and other glycoproteins (Shephard, 1993). The composition and rate of mucus secretion has been observed to change in response to microbial exposure or to environmental perturbation such as hyperosmolarity and acidity (Agarwal et al., 1979; Zuchelkowski et al., 1981; Ellis, 2001). The mucus substance secreted from the surface of fish performs a number of functions including disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding and nest building (Ingram, 1980; Fletcher, 1978). Despite an intimate contact with high concentrations of pathogens (bacteria and viruses) in their environment, the fish can still maintain a healthy system under normal conditions. This could be attributed to a complex system of innate defense mechanisms within themselves, particularly the products of broad spectrum-antimicrobial compound. Many researchers have proved that the mucus substances are good resistant to invading pathogens (Ingram, 1980; Fletcher, 1978; Austin and Mcintosh, 1988; Fouz et al., 1990).

Fish mucus (slime layer) is the first physical barrier that inhibits entry of microbes from an environment into fish. It acts as a chemical barrier containing enzymes and antibodies which can kill invading disease causing organisms (Rottmann et al., 1992). A fatty acid compositional study of the flesh of Haruan (*Channa striatus*) revealed those unusually high arachidonic acids, but almost no eicosapentaenoic acids, which were hypothesized to be actively involved in initiating tissue wound repair (Mat Jais et al., 1994). Antimicrobial activity in mucus has been demonstrated in several fish species (Austin and Mcintosh, 1988), yet this activity seems to vary from one fish species to the other and can be specific towards certain bacteria (Noga et al., 1995). When we are reviewing the literature among the fresh water fishes the studies are available mostly on cold water fishes.

Studies are available on *C. striatus*, *Cyprinus carpio* (Cole et al., 1997) and *Etheostoma crossopterum* (Knouft et al., 2003). Though studies are available on the microbicidal activities of fish mucus they are pertaining only against bacteria except a single study against fungi (Hellio et al., 2002). Antimicrobial activity was demonstrated in *Channa punctatus* and *Cirrhinus mrigala* (Kuppulakshmi et al., 2008). Antimicrobial activity of skin and intestinal mucus of five different freshwater fish *Channa* species was studied by Dhanraj et al. (2009). Apart from these no studies are available on the mucus of the cultivable fresh water fishes like *Catla catla, Labeo rohita* (Indigenous fishes), *Hypophthalmichthys molitrix* and *Ctenopharyngodon idella* (Exotic fishes).

Hence it was decided to evaluate the bactericidal and fungicidal properties of surface and column feeders namely *C. catla*, *H. molitrix, Labeo rohita* and *Ctenopharyngodon idella*. In the present investigation few microbial species of bacteria such as, *Klebsiella pneumonia, Vibrio cholerae, Salmonella typhi, Escherichia coli, Pesudomonas aeruginosa* and fungi*, Mucur globosus, Rhizopus arrhizus, Candida albicans, Aspergillus flavus* and *Aspergillus niger* were selected.

MATERIALS AND METHODS

Collection of mucus

The healthy live fishes approximately 6 months old, weigh about 500 gms of each *C. catla, L. rohita, H. molitrix C. idella* were purchased from near by fish farm in Pinnalur, Cuddalore District, Tamil Nadu. Mucus was carefully scraped from the dorsal surface of the body using a sterile spatula. Mucus was not collected in the ventral side to avoid intestinal and sperm contamination. The collected fish mucus was stored at 4ºC for further use.

Preparation of mucus sample for the antibacterial and antifungal studies.

The mucus samples were collected aseptically from the fish and thoroughly mixed with equal quantity of sterilized physiological saline (0.85% NaCl) and centrifuged at 5000 rpm for 15 min, the supernatant was used for the antimicrobial studies and kept at 4°C until use.

A thin layer of molten agar (Muller Hinton Agar) was dispensed in petriplates of 10 × 10 cm and was labled properly. Triplicates were maintained for each strain. In the same way for fungal studies PDA medium was dispensed in petriplates for different strains of fungi in triplicates and the plates were marked.

Inoculation of bacterial strains

The microbial strains were collected from the Balaji High-tech Laboratory in Manjakuppam, Cuddalore district, Tamil Nadu.

In vitro antibacterial assay was carried out by disc diffusion technique (Bauer et al., 1996). Whatman No.1 filter paper discs with 4 mm diameter were impregnated with known amount (10 µl) of test sample of fish mucus and a standard antibiotic disc. At room temperature (37ºC) the bacterial plates were incubated for 24 h. The fungal plates were incubated at 30ºC for 3 to 5 days for antifungal activity. The results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs were expressed in terms of diameter of

Table 1. Antibacterial activity of skin mucus from Catla and Silver carp.

S/N	Name of the Bacterial Pathogens	Zone of inhibition (in mm)		Control (Ciproflaxin) (in mm)
		Catla	Silver carp	
1	*K. pneumonia*	25	22	24
2	*V. cholerae*	21	20	22
3	*S. typhi*	32	15	28
4	*E. coli*	23	16	22
5	*P. aeruginosa*	29	22	32

Table 2. Antibacterial activity of skin mucus from Rohu and Grass carp.

S/N	Name of the Bacterial Pathogens	Zone of Inhibition (in mm)		Control (Ciproflaxin) (in mm)
		Rohu	Grass carp	
1	*K. pneumonia*	24	7	24
2	*V. cholerae*	21	7	22
3	*S. typhi*	14	12	28
4	*E. coli*	21	17	22
5	*P. aeruginosa*	19	15	32

zone of inhibition and were measured in mm using cm scale, recorded and the average were tabulated.

Antimicrobial assay

The spectrum of antimicrobial activity was studied using five different strains of human pathogenic bacteria and five species of fungal pathogens. One antibiotic agent Ciproflaxin for pathogenic bacteria and Ketoconazole for pathogenic fungi were used as control.

RESULTS

Antimicrobial effect of the mucus of surface feeder and column feeder freshwater fishes namely, *C. catla*, *H. molitrix* (Surface feeder), *L. rohita* (Column feeder), *C. idella* were tested against, pathogenic bacteria viz, *K. pneumonia*, *V. cholerae*, *S. typhi*, *E. coli*, *P. aeruginosa* and five pathogenic fungi viz, *Mucor globosus*, *Rhizopus arrhizus*, *Candida albicans*, *Aspergillus flavus, Aspergillus niger*. The activity was measured in terms of zone of inhibition in mm.

Antibacterial effect of mucus from surface feeders and column feeders

The inhibition effects of mucus of *C. catla, H. molitrix* against five pathogenic bacterial strains are given in Table 1 and the zone of inhibition by the mucus of *L. rohita, C. idella* are given in Table 2. The zone of inhibition values of mucus were compared with control (Ciproflaxin) and the observed values are tabulated in Tables 1 and 2, respectively.

The mucus of *C. catla* showed more effect in controlling the growth of gram-negative bacteria *Salmonella typhi* with an inhibition zone of 32 mm in diameter which is more than the control (Figure 3). Next to *S. typhi*, the mucus of *C. catla* showed a better effect on *P. aeruginosa* having an inhibition zone of 29 mm in diameter (Figure 5). That was followed by the *K. pneumonia* with an inhibition zone of 25 mm in diameter (Figure 1). Among the five gram-negative bacteria tested *V. cholerae* and *E. coli* showed very less sensitivity to the mucus of *C. catla* with an inhibition zone of 21 and 23 mm in diameter (Figures 2 and 4).

The mucus of *H. molitrix* showed more effect in controlling the growth of *K. pneumonia* and *P. aeruginosa* with an inhibition zone of 22 and 22 mm in diameter (Figures 1 and 5). Moderate effect was observed in controlling the growth of *V. cholerae* with a zone of inhibition is 20 mm in diameter (Figure 3). *S. typhi* (15 mm) and *E. coli* (16 mm) showed very less sensitivity to the mucus of *H. molitrix* (Figures 3 and 4).

Whereas the mucus of *L. rohita* showed a strong effect in controlling the growth of *K. pneumonia* with an inhibition zone of 24 mm diameter (Figure 1). *V. cholerae* and *E. coli* showed a better effect in the mucus of *H. molitrix* with an inhibition zone of 21 mm in diameter (Figures 2 and 4). Among the five bacteria tested *S. typhi* and *P. aeruginosa* showed less sensitivity to the mucus with an inhibition zone is 14 mm and 19 mm in diameter (Figures 3 and 5).

The mucus of *C. idella* showed more effect in controlling the growth of *E. coli* with an inhibition zone of 17 mm diameter (Figure 4). The moderate effect was observed in controlling the growth of *S. typhi* (12 mm) and *P. aeruginosa* (15 mm) by the mucus of *C. idella*.

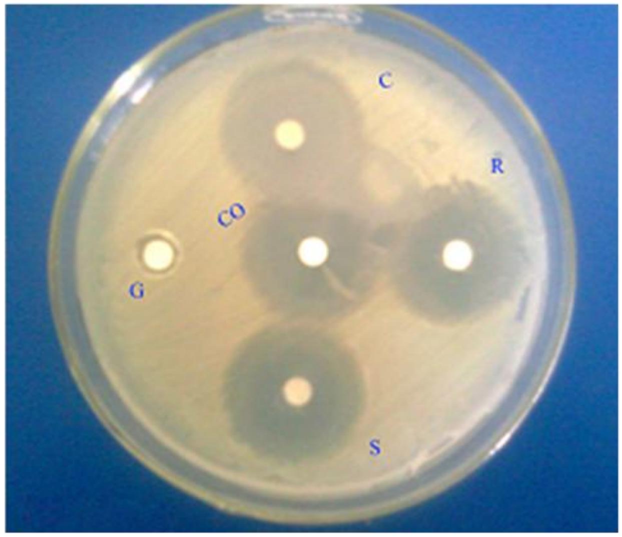

Figure 1. *K. pneumonia* antibacterial activity of fish skin mucus.

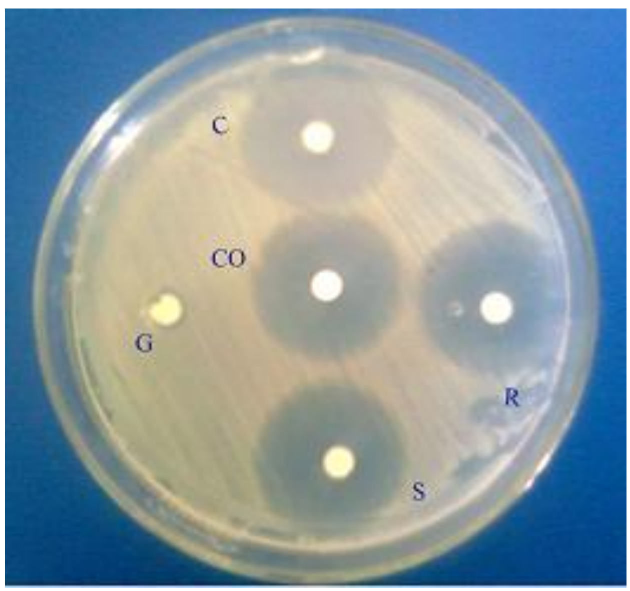

Figure 2. *Vibrio cholera* Antibacterial activity of fish skin mucus.

Among these, the *K. pneumonia* and *V. cholerae* showed very less sensitivity to the mucus of *C. idella* with an inhibition zone of 7 mm diameter (Figures 1 and 2).

The antibacterial activity of control Ciproflaxin showed maximum activity in three bacteria and some bacteria it showed less activity than the mucus sample.

Antifungal effect of mucus

The effect of mucus from *C. catla, H. molitrix* against five pathogenic fungal strains are given in Table 3 and the zone of inhibition by the mucus of *L. rohita, C. idella* are given in Table 4. The zone of inhibition values of control (Ketoconazole) are tabulated in Tables 3 and 4, respectively. The mucus of *C. catla* showed a maximum effect in controlling the growth of *A. flavus* with an inhibition zone of 17 mm diameter which is less than the control (19 mm) (Figure 9). Next to this, the *Mucor globosus* (16 mm) and *R. arrhizus* (16 mm) have more zone of inhibition (Figure 6 and Figure 7). Whereas as the mucus of *C. catla* has less effect in controlling the growth of *C. albicans* (14 mm) and *A. niger* (9 mm) (Figures 8 and 10). Likewise the mucus collected from *H. molitrix* has highest effect in controlling the growth of *A. flavus* with an inhibition zone is 17 mm (Figure 9). On the

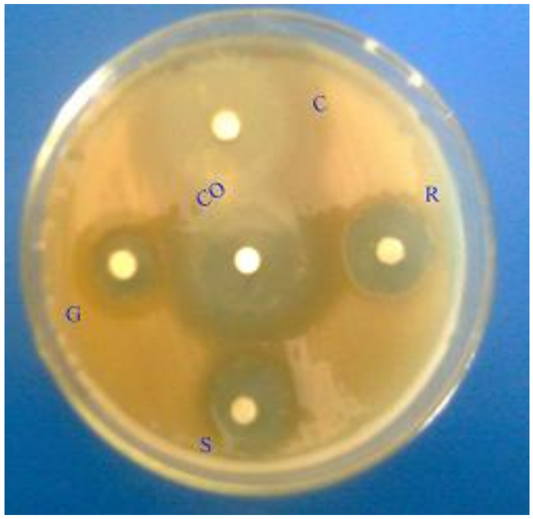

Figure 3. *Salmonella typhi* Antibacterial activity of fish skin mucus.

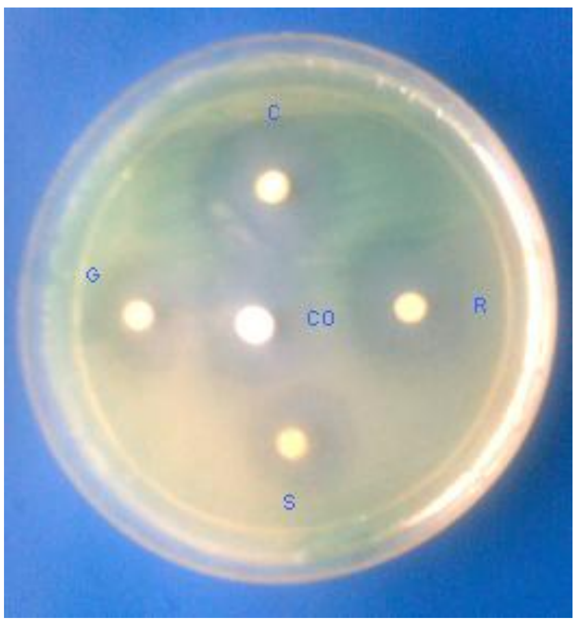

Figure 4. *Escherichia coli* Antibacterial activity of fish skin mucus.

contrary there is no effect in controlling the growth of *Rhizopus* sp and *C. albicans*. (Figures 7 and 8). The mucus of *H. molitrix* shows the moderate effect in controlling the growth of *Mucor globosus* (14 mm) and *A. niger* (8 mm) (Figures 6 and 10).

L. rohita a column feeder showed more effect in controlling the growth of *A. flavus* (17 mm). The mucus of *L. rohita* has very less sensitivity against the growth of *M. globosus* and *R. arrhizus* (14 mm) (Figures 6 and 7). But the mucus of *L. rohita* has the better effect in controlling the growth of *C. albicans* (15 mm) and *A. niger* (15 mm) (Figures 8 and 10).

The mucus of *C. idella* shows the highest activity against *A. flavus* with an inhibition zone of 16 mm in diameter (Figure 9). Next to *A. flavus* the mucus shows better effect in controlling the growth of *M. globosus* (15 mm), *R. arrhizus* (15 mm) and *C. albicans* (13 mm) in diameter (Figures 6, 7 and 8). But it failed to control the growth of *A. niger* (Figure 10).

The antifungal activity of control Ketoconazole showed a variety of activity against *M. globosus* (16 mm), *R. arrhizus* (15 mm), *C. albicans* (17 mm), *A. flavus* (19 mm)

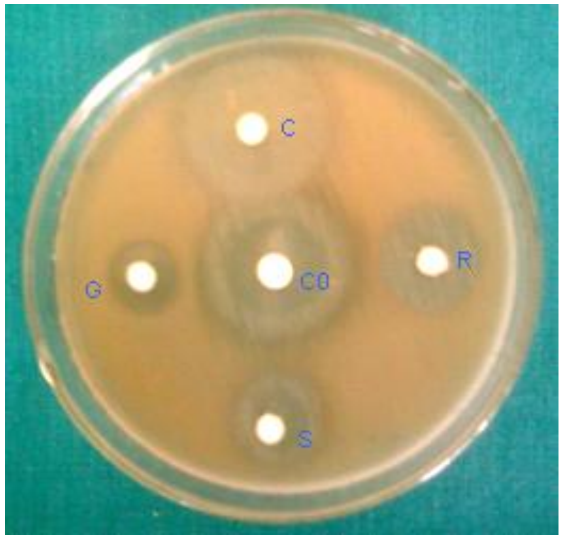

Figure 5. *Pseudomonas aeruginosa* Antibacterial activity of fish skin mucus. Co – Control, C – Catla, R – Rohu, S - Silver carp, G - Grass carp.

Table 3. Antifungal activity of skin mucus from Catla and Silver carp.

SNo	Name of the Fungal Pathogens	Inhibition (in mm)		Control (Ketoconazole) (in mm)
		Catla	Silver carp	
1	*M. globosus*		14	16
2	*R. arrhizus*	16	-----	15
3	*C. albicans*	14	-----	17
4	*A. flavus*	17	17	19
5	*A. niger*	9	8	11

Table 4. Antifungal activity of skin mucus from Rohu and Grass carp.

SN	Name of the Fungal Pathogens	Zone of Inhibition (in mm)		Control (Ketoconazole) (in mm)
		Rohu	Grass carp	
1	*M. globosus*	14	15	16
2	*R. arrhizus*	14	15	15
3	*C. albicans*	15	13	17
4	*A. flavus*	17	16	19
5	*A. niger*	15	----	11

and *A. niger* (11 mm), respectively.

DISCUSSION

The epithelial surfaces of fish, such as the skin, gills and the alimentary tract provide first contact with potential pathogens. The biological interface between fish and their aqueous environment consists of a mucus layer composing of biochemically-diverse secretions from epidermal and epithelial cells (Ellis, 1999). This layer is thought to act as a lubricant to have a mechanical protective function, to be involved in osmoregulation and play a possible role in immune system of fish. Fish tissue and body fluids contain naturally occurring proteins or glycoproteins of non-immunoglobulin nature that react with a diverse array of environmental antigens and may confer an undefined degree of natural immunity to fish. Antimicrobial peptides are among the earliest developed molecular effectors of innate immunity and are significant

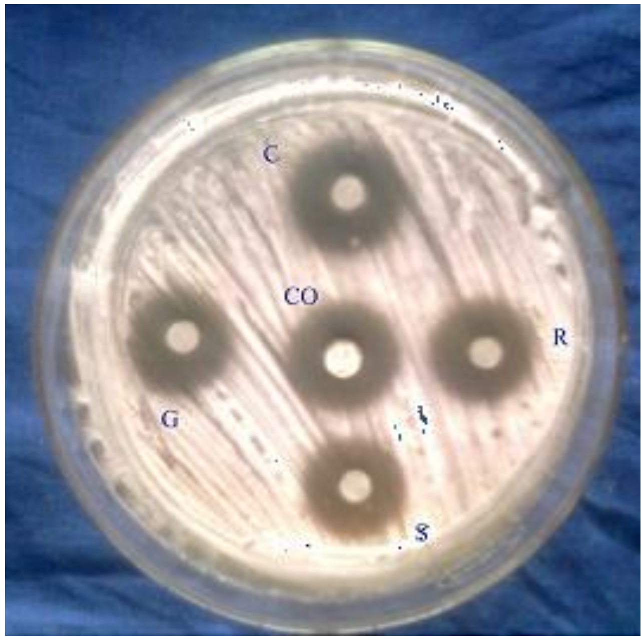

Figure 6. *Mucor globosus* Co – Control, C – Catla, R – Rohu, S - Silver carp, G - Grass carp.

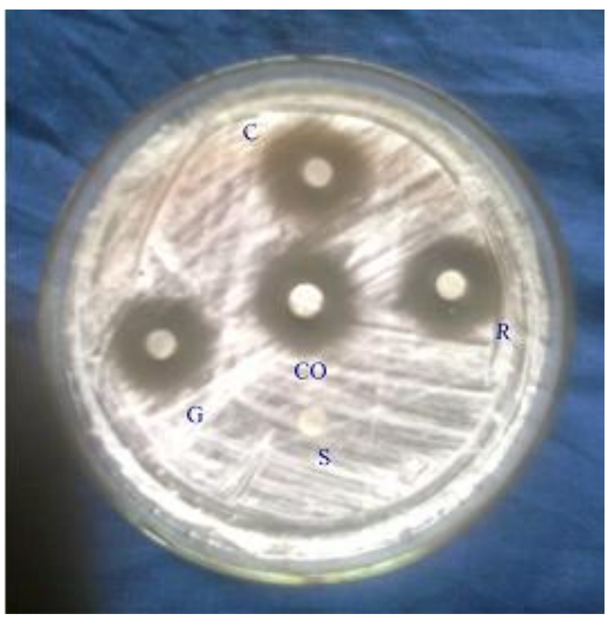

Figure 7. *Rhizopus arrhizus* Co – Control, C – Catla, R – Rohu, S - Silver carp, G - Grass carp.

in the first line of host defense response of diverse species.

Most antimicrobial peptides found through out the animal and plant kingdom are small, functionally specialized peptides (Boman, 1995). Several endogenous peptides with antimicrobial activity from fish, especially from the skin and skin mucus are reported (Park et al., 1997). Endogenous peptides play an important role in fish defense, possess broad spectrum of antimicrobial activity against bacteria, yeast and fungi. The epidermic and the epithelial mucus secretions act as biological barriers between fish and the potential

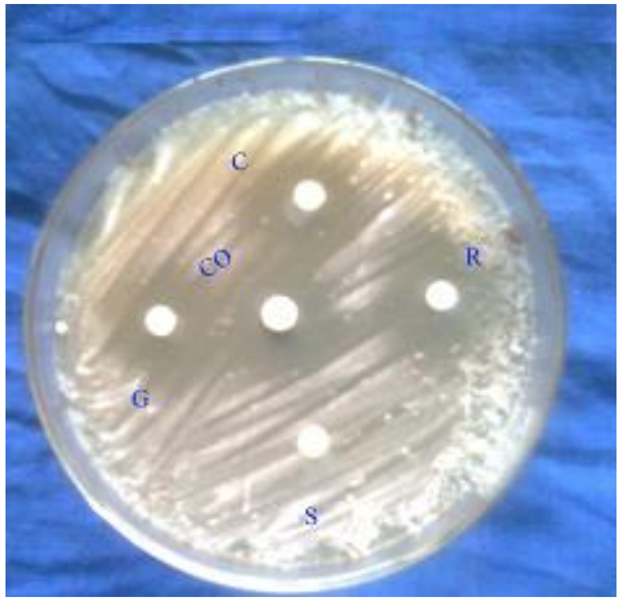

Figure 8. *Candida albicans* Co – Control, C – Catla, R – Rohu, S - Silver carp, G - Grass carp.

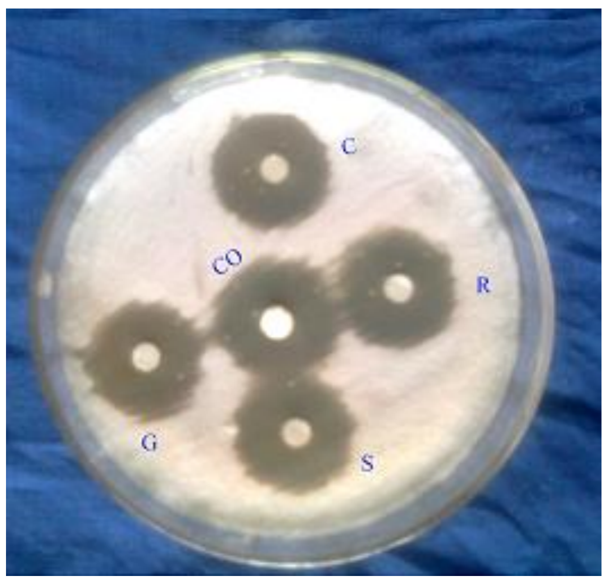

Figure 9. *Aspergillus flavus* Co – Control, C – Catla, R – Rohu, S - Silver carp, G - Grass carp.

pathogens of their environment (Shephard, 1993). Group of researchers suggest that the epidermal mucus acts as a first line of defense against pathogens and therefore may offer a potential source of novel antimicrobial compounds (Ellis, 2001; Fouz et al., 1990; Grinde et al., 1988; Nagashima et al., 2001; Sarmasik, 2002).

The mucus producing cells in epidermal and epithelial layers had been reported to differ between fish species and therefore could influence the mucus composition. Furthermore, the biochemical substances of mucus have been showed to differ depending on the ecological and physiological condition (Subramanian et al., 2008). In the present study also the mucus secreted by fishes are having strong resistance to the microbes. The mucus collected from all the four fishes show vary activity against the tested bacteria.

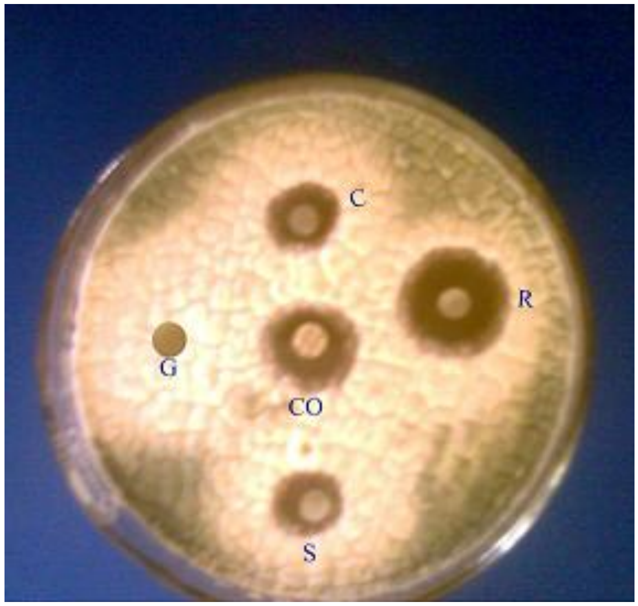

Figure 10. *Aspergillus niger* Co – Control, C – Catla, R – Rohu, S - Silver carp, G - Grass carp.

Amphipathic α-helical peptides, such as dermaseptin, ceratotoxin and magainin bind with anionic phospholipids-rich membranes and dissolve them like detergents (Pouny et al., 1992; Shai, 1995). These peptides are known to exert action by binding to the surface of the microbial membranes and causing a lysis of the intracellular contents. Our present study was also supported by the above studies in showing the antibacterial activity.

Fish contain serum and cellular interferon which possess anti-viral proteins, enzymes- inhibitors (e.g. α-macroglobulin and other β-globulins) that inhibit the extra cellular proteases secreted by pathogens (Alexander and Ingram, 1992). They added that number of relatively specific lytic molecules, like hydrolase enzymes (Lysozyme, Chinase and Chitobiase) act on fungi and bacteria. Fish also contain lectins possess antifungal and antibacterial activities. Mucus contain several proteases (serine proteases, cysteine proteases, metalloproteases and trypsin (like proteases) having strong antibacterial activity (Fast et al., 2002). The mechanism by which antimicrobial substance kill microbes are still unclear, but it is currently thought that different peptides employ different strategies. These include the fatal depolarization of the cell membrane (Westerhoff et al., 1989), the formation of pores and subsequent leakage of the cell contents (Yang et al., 2000) or the damaging of critical intracellular targets after internalization of the peptide (Kargol et al., 2001).

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. Few antimicrobial agents structurally identified in the mucus of bony fishes are proteins. It has been proposed that these compounds bind to and essentially dissolve cellular membrane (Ebran et al., 1999; Zasloff, 2002). The data of present study indicate that the antimicrobial activity of the fish mucus may be due to the presence of the above said substances. The mode of action of mucus is yet to be determined but studies have proposed various killing mechanisms for fish derived AMPs such as cytoplasmic membrane disruption, pore or channel formation (Syvitski et al., 2005) and inhibition of cell wall and nucleic acid synthesis (Partzykat et al., 2002; Brogden, 2005).

In the present study, variation in their antimicrobial activity was observed among the fish mucus. This may be due to the variation in the relative levels of lysozyme, alkaline phosphatase, cathepsin B and proteases of the epidermal mucus of all fish species (Subramanian et al., 2007).

Both the indigenous and exotic fish species have the activity against the bacterial pathogens, whereas some fungal pathogens were not controlled by the mucus of exotic fishes. But the mucus of indigenous fishes controls the tested fungal pathogens. Native fish species (indigenous) thrives better in prevalent conditions in controlling the mosquitoes than exotic fishes (Chandra et al., 2008). Falling in line with the above observation, the indigenous fish species such as *C. catla* and *L. rohita* show higher antimicrobial activity than that of the exotic fish species such as *H. molitrix* and *C. idella.* This is the first report on the antimicrobial activity of skin mucus of cultivable indigenous fishes of India. Moreover the mucus of fish possesses antimicrobial agents which could be used to formulate new drugs for the therapy of infectious diseases caused by pathogenic and opportunistic microorganisms. These properties of mucus suggest that it may be beneficial in aquaculture and human health-related applications. Further studies are needed to isolate the bioactive compounds (antimicrobial substances) from the mucus of these cultivable fish species and the mechanism of antimicrobial action.

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