Chapter I
3.1. INTRODUCTION

Immune responses of lower vertebrates such as teleost fish are significantly less sophisticated than those of higher animals. As an aquatic environment, fish have unique anatomical and physical characteristics. Fish live in intimate contact with an environment containing many organisms including both saprophytic and pathogenic microbes capable of digesting and degrading fish tissues (Ellis, 2001; Plouffe et al., 2005). Fish depend greatly upon innate or non-specific immune mechanisms for initial protection against infectious agents. Antibody and specific cell mediated responses in fish are less diverse than those of mammals (Ellis, 1982) and are also limited in response time by temperature constraints on fish metabolism (Avtalion et al., 1976, Bly and Clem, 1991). On the other hand due to the slow adaptive immune response of fish makes innate immunity, which is fast acting and temperature independent (Ellis, 2001), and predominant system of fish host defense.

The innate immune response is essential for the survival of this whole class of animals. Concisely, fish have evolved a number of innate immune responses to defend themselves against infection. The defense of innate immune system starts from the epithelial surfaces of fish, such as the skin, gills and alimentary tract, provide first contact with potential pathogens. These surfaces of above organs were composed of living, non-keratinized tissue covered by a layer of mucus which contains a number of non-specific antimicrobial factors, such as lysozyme, transferrin and C-reactive protein (Roberts, 1989). The host defense of fish includes many elements such as antimicrobial peptides (Cole et al., 1997) polypeptides (Fernandes et al., 2002), non-classical complement activation, release of cytokines, inflammation and phagocytosis (Ellis, 2001; Magnadottir, 2006). In skin the multiple layers of keratinocytes at different stages of differentiation comprise the epidermis. Basal keratinocytes, which are attached to the basement membrane, are undifferentiated proliferative cells. As keratinocytes differentiate upwards to form the permeability barrier of skin, both their morphology and gene expression profile change. The spinous and granular layers lie directly above the basal keratinocytes and are covered by the stratum corneum, which consists of nonviable, terminally differentiated anucleated cells (Haake et al., 2003). Secretory
granules such as lamellar bodies form in these differentiated epidermal layers. Lamellar bodies transport lipids and hydrolytic enzymes involved in stratum corneum formation and have been shown to store cytokines, including TNF-α and IL-1 (Nickoloff, 1999; Oren et al., 2003), antimicrobial peptides such as β-defensins and cathelicidins (Oren et al., 2003; Braff et al., 2005). The expression of AMPs differs depending on the cell and tissue type, but in most cases AMPs are co-expressed as groups that act together. For example, in skin, more than 20 antimicrobial peptides and proteins have been identified (Braff and Gallo, 2006), including cathelicidins, β-defensins and others. A few reports only available on the immunological functions of the skin peptides, and it is unlikely that the immunomodulatory actions of the mammalian system.

Antimicrobial peptides produced by epithelial cells and granulocytes kill pathogens and stimulate additional host defense mechanisms.

Antimicrobial peptides contribute importantly to skin defense, particularly when physical barriers of innate immunity have been breached. In response to cutaneous injury or infection, antimicrobial peptides are synthesized or deposited by various cells, including keratinocytes and neutrophils. Upon activation, the antimicrobial peptides contribute to host defense against pathogenic infection by inhibiting the growth of potentially harmful microbes and by stimulating additional immune functions such as chemotaxis. In skin the multiple layers of keratinocytes at different stages of
differentiation comprise the epidermis. Basal keratinocytes, which are attached to the basement membrane, are undifferentiated proliferative cells. The keratinocytes differentiate upwards to form the permeability barrier of skin, both their morphology and gene expression profile change. The spinous and granular layers lie directly above the basal keratinocytes and are covered by the stratum corneum, which consists of nonviable, terminally differentiated anucleated cells (Haake et al., 2003). Secretory granules such as lamellar bodies form in these differentiated epidermal layers. Lamellar bodies transport lipids and hydrolytic enzymes involved in stratum corneum formation and have been shown to store cytokines, including TNF-α and IL-1 (Nickoloff 1999; Oren et al., 2003), antimicrobial peptides such as β-defensins and cathelicidins (Oren et al. 2003; Braff et al., 2005). The expression of AMPs differs depending on the cell and tissue type, but in most cases AMPs are co-expressed as groups that act together. For example, in skin, more than 20 antimicrobial peptides and proteins have been identified (Braff and Gallo, 2006), including cathelicidins, β-defensins and others. AMP’s exhibit multiple functions related to their capacity to disrupt membranes. They have the ability to confer protection against a variety of pathogens and the potential to act as cytotoxic agents against certain type of cancers. However, the direct antimicrobial activity implicit to the term ‘AMP’ has strongly influenced the elucidation of the function of these peptides as ‘natural antibiotics’. Some AMPs can confer protection by an indirect mechanism. They were not simply because they can kill microbes. But potent immune regulators, altering host gene expression, acting as chemokines and/or inducing chemokine production, inhibiting Lipopolysaccharide or hyaluronan induced pro-inflammatory cytokine production, promoting wound healing and modulating the responses of dendritic cells or T cells of the adaptive immune response. AMPs were acting as a bridge between innate and adaptive immunity. All of these functions favor resolution of infection, reverse potentially harmful inflammation, and complement the direct antimicrobial action.

The Antimicrobial peptides have several mechanisms to kill the pathogens by which different classes of AMPs could act on the microbial target membrane (Brogden, 2005). In the ‘carpet model’, peptides accumulate on and orient parallel to the membrane surface. At certain high concentration of peptides, AMPs disrupt the bilayer membrane in a detergent-like manner, resulting in the formation of micelles and leakage of cellular contents. The ‘toroidal-hole model’, the polar heads of the peptides
face the polar head groups of the lipids, inducing the lipids to form a continuous bend from the top to the bottom in the fashion of a toroid which is lined by both peptides and the lipid head groups. But some of the cationic AMPs adopt an $\alpha$-helical configuration, attach to, aggregate and insert into oriented bilayers that are hydrated with water vapor, leading to the formation of ‘barrel-stave’ holes in the membrane. This model, the hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore. Notably, the differences between the toroidal model from the barrel-stave model is that peptides in the former are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer. Finally, although most AMPs have been shown to disrupt cell membranes and induce microbial killing, a few AMPs kill bacteria without any detectable lysis. These AMPs can penetrate the cell membrane and bind to different targets, such as DNA, to inhibit bacterial growth.

Hence the present chapter focused on the innate immune defense of the first line barrier present in the Gill, Liver, Intestine, Kidney and skin crude peptide extracts from catfish *Clarius butrachus* against *E.Coli* and then the active extract among various organs choosed and screened for antimicrobial activity against Human and veterinary pathogens. From the literature, natural peptides were shown to be effective against the Gram-negative and Gram-positive bacteria, by inhibiting the growth of microorganisms. Therefore the present work deliberates on identification of novel antimicrobial peptides that might be a good therapeutic alternative for the treatment of fish and Human diseases (Hultmark et al., 1997).

### 3.2. MATERIALS AND METHODS

#### 3.2.1. Collection of animals

The growing live healthy fishes approximately 6 months old, weight about 200-500gm each *Clarius butrachus* were purchased from the Lake located at Navalpattu near, Matthoor, Tiruchirappalli.

#### 3.2.2. Acclimatation

The purchased fishes were acclimated for 10 days in laboratory using tap water. After 10 days they were maintained in flow-through freshwater tanks (28± 2 °C) and fed daily *ad libitum* with commercial fish pellets.
3.2.3. Collection of various organs from fish

The fishes were anaesthetized with MS222. Then it was carefully dissected the various organs like Liver, Intestine, Kidney, and Skin were collected. The collected organs were buffered with PBS and stored at 4 °C for further use.

3.1.4. Estimation of proteins

Preparation of Peptide extracts from *C. butrachus*

Fish measuring 15 to 20 cm in length and 250 to 500 g in weight were chosen and transported to the laboratory. The epidermis was scrapped from the dermis with a scalpel from the *Clarius butrachus* and other organs like Liver, Intestine and Kidney was carefully dissected and stored in sterile vial at – 80 °C. 500mg of each organ was homogenized separately with 50mM Tris buffer pH 7.2 and it was homogenized with electronic homogenizer for 3 to 5 min. Homogenate was centrifuged at 16,000 rpm for 45 min at 4 °C. Then the supernatant were collected and stored at – 80 °C until use. The protein was collected and the concentration was determined by adapting the method of Bradford (1976) using Bovine Serum Albumin (BSA) as standard.

Principle

Coomassive Brilliant Blue G250 was used for quantification of protein in solution. The dye and the protein form a complex by a non-covalent bond to form the non-covalent compound. The intensity of the protein-dye complex was read at 595 nm in a spectrophotometer.

Reagents

*Bradford reagent*: 100 mg of Coomassive Brilliant Blue G250 was dissolved in 50 ml of 95% ethanol and to this 100 ml of 85% ortho phosphoric acid was added. The resultant solution was diluted to a final volume of 1 litre.

Procedure

The standard bovine serum albumin (BSA) solution (stock solution = 1 mg/ml) of varying series of concentration (20 µg to 100 µg) was taken and made up to 1 ml using distilled water in test tubes. The unknown samples, 100 µl, were taken and then made up to 1 ml with distilled water. To all the tubes 2.5 ml of Bradford’s reagent was added. After 2 minutes, optical density (O.D.) at 595 nm was read using a ultra violet-
visible (UV-vis) spectrophotometer (Perkin-Elmer, USA) and standard graph was plotted where x axis represented the concentration of standard and y axis, the O.D. of the standard. From this graph the protein concentration of unknown sample was calculated and the volume to be loaded in the SDS-PAGE was decided upon.

**Calculation of protein concentration:**

\[
\text{Concentration of protein of the sample} = \frac{\text{Optical density of the Sample}}{\text{Optical density of the standard}} \times \text{Protein Concentration of Standard}
\]

3.2.5. Preparation of media for antibacterial studies

*In vitro* antibacterial activity of fish was studied against bacterial strains which are designated as human pathogens. Agar well diffusion method was adopted 28gms of Nutrient Agar (Hi-media, India) was mixed with 1000ml of distilled water in a conical flask and sterilized in an autoclave. The molten medium was then dispensed into 5 conical flasks and was kept in laminar flow chamber aseptically until use.

**Composition of Nutrient Agar (gm/l)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptides digest of animal tissue</td>
<td>5.00</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.00</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Casein acid hydrolysate</td>
<td>17.50</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.50</td>
</tr>
<tr>
<td>Agar</td>
<td>15.00</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 ± 0.2</td>
</tr>
</tbody>
</table>

**Plating of agar**

Petri dishes measuring about 90x 15mm were sterilized in an autoclave and used for the antimicrobial studies. A thin layer of molten agar (Nutrient Agar) was dispensed in 10x3 petriplates (for 10 different bacterial strains in triplicate) and was labeled properly.

**Inoculation of bacterial strains**

Seven pathogenic bacterial strains namely *Escherichia coli, Klebsiella pneumoniae, Streptococcus aureus, Staphylococcus aureus, Aeromonas hyrophilla,*
Protease vulgaris and Pseudomonas putida were used. They were swabbed onto the respective petriplates. 18 hours old cultures in logarithmic phase of growth were used for swabbing.

**Filling the wells with samples**

100µl of samples from C. butrachus were dispensed separately in two wells made in petriplates.

**Incubation of petriplates**

The bacterial cultures were incubated at 37 ºC for 24 hours. The activity was evaluated by measuring the radius (r in mm) of the inhibition zones. For each and every bacterium the inhibition zones in triplicates were noted down and the pooled average was given in the table 2 to 8 for the bacteria.

**3.2.3.6. Antimicrobial assay**

The antimicrobial assay was performed by viz. Agar well diffusion method (Perez et al., 1990). The Nutrient Agar was inoculated with the 100 µl of the inoculums (1 x 108 CFU) and poured into the Petri plate (Hi-media). For agar well diffusion method, the disc (0.7cm) (Hi- Media) was saturated with 100 µl of the test compound, allowed to dry and was introduced on the upper layer of the seeded agar plate. The well was formed with the help of a cork-borer (0.85cm). 100 µl of the extract was introduced into the well. The plates were incubated overnight at 37°C. Microbial growth was determined by measuring the radius of zone of inhibition. For each bacterial strain controls were maintained. Tris buffer were used as negative control and Tetracycline was used as positive control.

**3.2.4. Determination of the effective 50% reduction concentration (EC50)**

The effective 50% reduction concentration (EC50) by skin peptide extracts on each of the pathogens was depicted in table-1. It was performed using the microtitre broth dilution assay (Friedrich et al., 1999) The concentration of the peptides extract were quantified using Bradford’s method. Various concentration of peptide were taken viz. 50, 75, 100, 125, 150, 175, 200, 225, 250µg for assessing the EC50. One hundred microlitres of each bacterial suspension containing 105 colony forming units (CFU) per ml was mixed with serially known concentration of 50-250 µg test peptide was mixed
with serial in 900 Microliter of Muller Hinton broth in sterile tube and then 300µl were transferred into polypropylene 96-well microtitre plates. The positive control well contained bacteria and broth only. The Microtiter plates were incubated and the optical density (OD) was read at 570nm using an Biotech synergy HT microtitre plate reader, U.S against a blank comprising broth only. Values for experimental wells were recorded when the OD reached 0.2 in the positive control well. The EC50 was considered to be the lowest concentration of protein that reduced the growth by 50% relative to the control. The minimal bactericidal concentration (MBC) was obtained by plating out the contents of each well showing no visible growth. MBC was taken as the lowest concentration of protein that prevents any residual colony formation after incubation for 24 h at the appropriate temperature. Amoxicillin (Ranbaxy) pharmaceuticals were used as reference.

3.3. RESULTS

3.3.1. Protein concentration

The concentrations of total protein at which the first line innate defense system of various organs such as the Skin, Liver, Kidney and Intestine were estimated. Among them liver (420 ±0.62mm) and the Skin (385±0.46mm) were significantly increased than the Intestine (340±0.58mm) and Kidney (247±0.72mm) (Fig. 58). More interestingly, there was almost similar in the protein concentration between Intestine and the Skin of catfish.

3.3.2. Screening of In vitro defense activity of Crude peptide extract from various organs against Clinical strain E.coli.

The in vitro antibacterial activity of crude peptides present in the first line barrier of innate immune system was analyzed against the clinical pathogen E.coli (Fig. 2). The skin peptide extracts formed highest zone of inhibition (1.6±0.026mm) against E.coli that the other extracts. The kidney extracts exposed next highest activity (1.0±0.17mm). Compared to the above two extracts the Liver and the Intestine possesses more or less similar antimicrobial activity against the clinical pathogen E.coli. Whereas the liver and intestine exhibit almost similar level of inhibition (0.7±0.23mm and 0.6±0.14mm respectively) (Table.2).
3.3.3. *In vitro* Antimicrobial activity of Skin Peptide extracts against Human and Veterinary pathogens

Based on the above result the skin peptide respond good in defense activity against the clinical pathogen *E.coli*. The skin peptide extract was chosen for further studies. The skin extract was evaluated for the antibacterial activity against variety of pathogens. Seven pathogenic bacterial strains namely *Citrobacter*, *Klebsiella pneumoniae*, *Streptococcus aureus*, *Staphylococcus aureus*, *Aeromonas hyrophila*, *Proteus vulgaris* and *Pseudomonas putida* were used for antibacterial activity. Amid the bacterial pathogens the *Citrobacter*, *Aeromonas hydrophilla*, *Pseudomonas putida*, *Klebsiella pneumoniae*, *Streptococcus aereus* and *Staphylococcus aureus* exhibited pronounced inhibition indicated in the Fig. 3. Moreover the skin crude peptide extract was also screened for antifungal activity against fungal strains. The *Candida albicans*, *Candida trophicans*, *Cryptococcus neoformis* and *Aspergillus niger* were appraised for the antifungal activity of the crude skin peptides. Among them the *Candida albicans* (Fig-4) exhibit pronounced inhibition than other fungal pathogens. The zone of inhibition determined at 100 µg concentration of skin crude peptides against the all bacterial and fungal pathogens. The fish pathogen *Aeromonas hydrophila* exhibited maximum activity of 2.4±1.20 mm among the bacterial pathogen whereas the fungal pathogen *C.albicans* exposed showed highest activity 2.5±0.82mm. Subsequently the *C.bacter* (1.5 ±0.52), *Klebsiella pneumoniae* possess 1.0±0.68mm, demonstrates the moderate inhibition and the *Streptococcus aureus* (0.8±0.41),*Pseudomonas putida* (0.7± 0.34) and *Staphylococcus aereus* (0.5±0.19) exhibits minimum inhibition (Fig. 5).

3.3.4. Effective 50% reduction concentration (EC50) of skin peptide against clinical isolates

The effective concentration (EC50) (estimated concentrations resulting in 50% survival) was assessed for the skin peptide extract against the various bacterial and fungal pathogens. the micro plate assay of EC50 and minimal inhibitory concentration exhibited significant activity of effective concentration assessed by log profit analysis displayed in the Table.1. The *K. pneumoniae* exposed the efficient activity possess lowest minimal inhibitory concentration of 15µg with EC50 of 81.65 µg, *C.bacter* 19µg with EC50 of 90.18 µg, *E.coli* (20µg with EC50 of 92.85µg), *A.hydrophila* (20µg with EC50 of 93.75µg), *P.vulgaris* (23µg with EC50 of 81.65 µg) and *C.albicans* (25µg with EC50 of 122.40µg). But the *Staph.aureus* (40µg with EC50 of 81.65 µg),
Str.aerues (15µg with EC30 of 133.03 µg) and P.putida (30µg with EC50 of 132.14 µg). The degree of EC50 was in the order of K. pneumoniae < C.bacter < E.coli < A.hyrdophila < C.albicans < P.vulgaris < P.putida < Str.aerues < Sta.aerues.

**Table.1**

<table>
<thead>
<tr>
<th>Clinical Isolates</th>
<th>Minimal Inhibitory Concentration (in µg)</th>
<th>Effective Concentration (EC50 in µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli</em></td>
<td>20</td>
<td>92.85</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>40</td>
<td>187.50</td>
</tr>
<tr>
<td><em>Streptococcus aereus</em></td>
<td>30</td>
<td>133.03</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>15</td>
<td>81.65</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>23</td>
<td>129.46</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>30</td>
<td>132.14</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>20</td>
<td>93.75</td>
</tr>
<tr>
<td><em>Citrobacter</em></td>
<td>19</td>
<td>90.18</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>25</td>
<td>122.40</td>
</tr>
</tbody>
</table>

**Fig. 1.** Estimation of Protein in various organs of *C.butrachus*

In the above Fig. the various organs extract like Skin, Intestine, Liver and Kidney were appraised for the Protein concentration using Bradford method.
Fig. 2 Antimicrobial activity of crude peptide extracts of various organs on *E. coli*

L = Liver  K = Kidney  I = Intestine  S = Skin  P = Positive Control  N = Negative Control

In the Fig. A) Liver  B) Intestine  C) Skin  and D) Kidney protein extracts were screened for its antibacterial activity against *E. Coli*, P-Positive control Tetracycline and N-Negative control the extraction buffer 50mM Tris was used. 100µl of various protein (L, S, K and I extracts were treated, Positive control 10mg/ml was added and 100 µl of Buffer were added in Negative control.
Table 2. Antimicrobial activity of crude peptide extracts of various organs on *E. coli*

<table>
<thead>
<tr>
<th>Source for Peptide extract</th>
<th>Zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>1.6 ± 0.026mm</td>
</tr>
<tr>
<td>Liver</td>
<td>0.7 ± 0.23mm</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.0 ± 0.17mm</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.6 ± 0.14mm</td>
</tr>
</tbody>
</table>

The antibacterial activity of various organ protein extracts of *C. butrachus* against a clinical pathogen *E. coli* was evaluated by agar well diffusion method. The active innate defense factors in the peptide extract were extracted with 50mM Tris buffer pH 7.4. The peptide extracts of different organs were treated as 100µl. Positive control wells were filled with the standard drug tetracycline. The negative control wells were filled with the Tris buffer. Each value represents the mean of five duplicates observations were expressed in ± S.D.
The skin peptide extract was screened for antibacterial activity against clinical pathogens *Aeromonas hydrophila, Pseudomonas putida, Citrobacter* and *E.coli*. Using agar well diffusion method. Three well were bored viz., S-Skin, P-Tetracycline and N-Extraction Buffer. Skin protein extract 100µg concentration was used and 10mg/ml tetracycline was used as positive control and 100µl Extraction buffer was used.
Fig. : 4 Antifungal Activity of Skin peptides against *Candida albicans*

Zone of inhibition at concentration 100µl
P=Positive control, N=Negative control, S=Skin peptides

Zone of inhibition of skin peptide extract against fungal pathogen such as *C. albicans* using agarwell diffusion method. The three well were bored viz., S-Skin, P-flucanozole extract 100µg concentration was used and 10mg/ml flucanozole was used as positive control and 100µl Extraction buffer was used.

Fig. : 5 Antimicrobial activity of Skin peptide from skin of catfish.

Zone of inhibition of skin peptide extract against Bacterial and fungal pathogens such as *K. pneumoniae, Aeromonas hydrophila, Pseudomonas putida, S.aerues, Str.aerues, Citrobacter* and *E.coli*. using agarwell diffusion method. The three well were bored viz., S-Skin,P-Tetracycline n extract 100µg concentration was used and 10mg/ml tetracycline was used as positive control and 100µl Extraction buffer was used.
3.4. DISCUSSION

Fish is one of the aquatic organisms that have managed to survive amidst of millions of pathogenic organisms (Pickering 1974). Microorganisms are small but become a very profound damage in human and animals. Due to the emergence of antibiotic-resistant bacteria, and the scarcity of new classes of useful antibiotics, there is an increasing need to identify novel antimicrobial peptides from various sources for the development of alternative therapeutants (Patrzykat and Douglas, 2003). The biological interface between fish and their aqueous environment consists of a mucus layer composed of biochemical diverse secretions from epidermal and epithelial cells (Ellis 1999). Moreover the mucus and epidermal layer of fish skin act as a lubricant (Rosen and Cornford, 1971), to have a mechanical protective function (Cameron and Endean, 1973), to be involved in osmoregulation and locomotion to play a possible immunological role (Fletcher, 1978) and to have some function in intra-specific chemical communication (Saglio and Blance, 1989). Therefore the antimicrobial compounds have been found associated with and dispersed from the epithelial mucus screening cell of tissue (Cole et al., 1997). Highest concentration of mucus cells should increase the amount of antimicrobial compound delivered to the skin surface. Many reports demonstrates that epithelial tissues produced antimicrobial molecules which serve as the first life defense against microbial invasion in a variety of vertebrates including humans (Ganz T. Defensive 1999). Some of the antimicrobial peptide of skin secretions and epithelial cells of rainbow trout play a vital role exposed defense against intracellular or extracellular pathogens (Fernandes and Smith, 2002).

The healing of human ailment by using therapeutics that are obtained directly from animals or ultimately are derived from these is known as zootheraphy animals based medicines have been elaborated from parts of the animal body or from product of its metabolism (corporal secretions and excrements), or from non-animal matters (Nest and cocoons). Fish by products are rich in potentially valuable proteins, minerals, enzymes, pigments or flavors (Hello et al., 2002). Antimicrobial compounds were mainly expressed as proteins reported in the variety of fishes e.g. Oncorhyncus mykiss (Kustin and McIntosh, 1988) Cyprinus carpio (Cole et al., 1997), Pleuronectes americanus (Lemaitre et al., 1997). Morone saxatilis, Chrysops hybried (Silphaduang and Noga, 2001) have been studied. These species are members of different orders (Salmoniformes, Cypriniformes, Pleurenciformes and Perciformes, respectively)
suggested that the presence of antimicrobial compounds in fish protein is not phylogenetically constrained and such compounds may be ubiquitous present among fishes. Richards et al., 2001 identified antimicrobial activity in peptide extracts of Atlantic Salmon from the liver, intestine, and skin. Trailing by this similar work the present study was aimed to screen the defense (antimicrobial) activity of Skin, liver, kidney and Intestine crude peptide extracts cat fish. The occurrence and localization of such antimicrobial substances, which may serve as a non-specific defense against bacteria, fungi, and algae as well as against ectoparasites were, until now only known for haired marine mammals with typical skin glands, like in the pinnipeds (Meyer et al. 2003). So to localize the strong defense activity of antimicrobial substances present in the various organs, the present study was planned to screen the efficacy of crude peptide extracts of Skin, liver, kidney and Intestine were evaluated in vitro using clinical pathogen Escherichia coli. Among the four different extract, the skin and kidney showed pronounced inhibition against pathogenic microorganism which may be identified as strong antimicrobial proteins. Amide the two extracts the skin demonstrates prominent activity. The antimicrobial activity was well connected with the action of pore forming properties against several pathogenic strains and these suggested that fish secrete antibacterial proteins, able to permeable the membrane of the target cell and thus act as a defense barrier (Haug et al., 2002). Hence the skin peptide extract was appraised against 7 pathogenic bacteria such as Klebsiella pneumoniae, Streptococcus aureus, Staphylococcus aureus, Aeromonas hydrophilla, Escherichia coli, Proteus vulgaris and Pseudomonas putida and a fungi Candida albicans. The above pathogens were pronouncedly inhibited by the skin crude peptides (Lancini et al., 1995). In comparison with this discussion, the present study emphases that the pronounced inhibition was done by the crude peptides in in vitro assay are referred as antimicrobicidal peptides similar to found amphipathic cyclic cationic antimicrobial peptides that have higher permeability and lysis activity against bacterial and fungal pathogens achieved strong antimicrobial effect (Muhle & Tam, 2001). The protective effects of the peptides have been attributed, in part, to the direct antimicrobial killing properties of purified peptides against bacterial, fungal or viral pathogens observed in vitro (Hancock and Diamond, 2000; Zasloff, 2002 and Bader et al., 2003). Most active antimicrobial peptides are able to interact with bacterial membranes, as described by four separate models (Hancock and Rozek., 2002). With substantial local perturbation of the cytoplasmic membrane bilayer, ion-permeable channels are created, leading to
increased cytoplasmic membrane permeability and bacterial cell death Ebran et al.,  
(1999) showed that the hydrophobic components of crude epidermal mucus of fresh-
water and sea-water fish exhibit strong pore-forming properties which were well 
 correlated with antibacterial activity. They have isolated novel glycosylated proteins 
from the hydrophobic supernatant of tench (Tinca tinca), eel (Anguilla anguilla) and 
rainbow trout (Oncorhynchus mykiss) mucus. The study of their secondary structure 
was performed by circular dichroism and revealed structures in random coil and α-helix 
in the same proportions. When reconstituted in planar lipid bilayer, they induced the 
formation of ion channels. This pore-forming activity was evident to possess a strong 
antibacterial activity. The present study was performed to find out the antimicrobial 
activity and identify the defense proteins. The present study was the first discovery to 
discover the novel antimicrobial peptides in Clarius butrachus, it has led to the most 
encouraging finding of in vitro efficacy of certain antimicrobial peptides against 
bacterial infections. Similar study was previously shown that 2 synthetic antimicrobial 
peptide derivative of naturally occurring insect peptide cecrupin-b had significant in 
vitro activity against bacterial pathogens of fish (Kely et al., 1990).

The MIC of moronecidin against S.iniae (1.25–2.5 µM ) obtained is similar to 
the MICs of β-lactam antibiotics (Weinstein et al., 1977). Followed by this report, the 
Effective concentration (EC50) and the Minimal inhibitory concentration of skin 
peptide extract were evaluated by in vitro antimicrobial activity with the positive 
control antibiotics tetracycline. The K. pneumoniae demonstrated a strong response 
than the other microbes. The degree of EC50 was obtained in the order of K. 
 pneu moniae < C.bacter < E.coli < A.hydrophila < C.albicans < P.vulgaris < P.putida < 
Str.aerues < Sta.aerues. All previously characterized fish AMPs, including 
pleurocidin (winter flounder), hagfish intestinal antimicrobial peptides, misgurin 
(mudfish), pardaxin (sole), and parasin (catfish), were found in cutaneous or epithelial 
mucosal layer. Similarly the crude antimicrobial peptide extract of non-specific defense 
line were strongly confirmed their defense activity against variety of pathogens 
(Bacterial and Fungal) from catfish C但不限于 further the unknown peptides should 
identified for the target defense molecules for many multi-drug resistant pathogens.