Journal for ReAttach Therapy and Developmental Diversities
eISSN: 2589-7799
2023 September; 6(9s): 2132 -2145

# Evaluation Of The Antimicrobial Activity Of Lyophilized Protein Extracts From Stomopneustes Variolari By Using Sds-Page

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**ABSTRACT**

Bioactive peptides, including antimicrobial polypeptides, lipids, and fish oil, are natural barriers to bacterial infections. These peptides increase permeability or destabilize membranes, but pathogens have developed countermeasures. Free fatty acids (FFAs) are a prominent source of antimicrobial compounds, with their spectra of action and potencies influenced by their structure and shape. Fish oil, found in marine animals, has various bioactivities, including anti-inflammatory action and increased survival for people with auto-immune diseases. A study evaluated the antimicrobial activity of lyophilized protein extracts from *Stomopneustes variolaris* using a well diffusion method. The antibacterial activity was assessed using gram-positive bacteria and gram-negative bacteria, while the antifungal activity was assessed using fungi. The results were analysed using one-way analysis of variance (ANOVA) and regression-correlation analysis using SPSS 10.0 software, with a 'P' value less than 0.05 considered as significant difference.

**Keywords :** Natural barriers, Bacterial infections, ANOVA, (FFAs) Free fatty acids, Bioactive peptides.

## INTRODUCTION

Uncovering the structural, compositional, and sequential characteristics of bioactive peptides has received a lot of interest lately (Lordan et al., 2011). Bioactive peptides, with 3-20 amino acid residues, are natural barriers to bacterial infections [1]. They are divided into four classes: antimicrobial polypeptides, linear peptides with α-helical domains, linear peptides with repetitive sequences, and cyclic peptides with disulfide bridges [2]. The mechanism of action of AMPs is to increase permeability or destabilize membranes. Pathogens have developed countermeasures to limit AMPs' effectiveness, such as chemical modifications or energy-dependent pumps at the membrane level. Intracellular bacterial pathogens have less effective resistance-limitation against cationic peptide-driven antimicrobial activity. Most peptides have a net positive charge and amphipathic nature, allowing them to persist at water-lipid interfaces [3].

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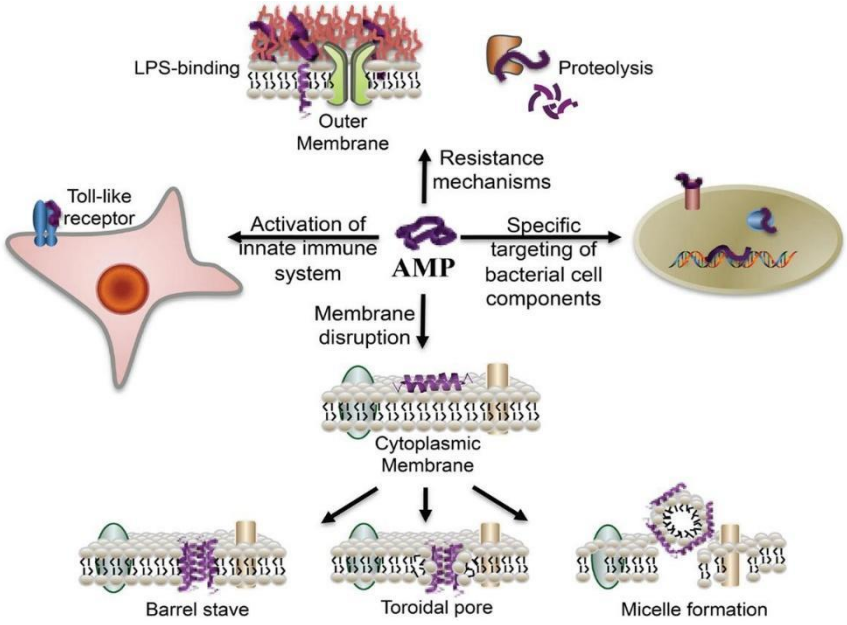


**Figure : 1. Overview of the broad spectrum of cellular interactions associated with antimicrobial peptides. In addition to the exerting antimicrobial activity by disrupting bacterial membranes, peptides may also bind to specific target proteins within microbial cells and activate the innate immune system. The binding of peptides to cell-surface LPS molecules and proteolysis contribute to bacterial resistance to AMPs (Adapted from Marsh et al., 2009).**

**Mechanism of Antimicrobial Peptides**

Antimicrobial peptides have a complicated mode of action, which makes it challenging to forecast their cytotoxic and antimicrobial effects. Certain peptides exhibit tumour cell selectivity and have plausible actions in animal models without conspicuous toxicity. To overcome this problem, an assay was created that measures the impact of peptides on the trans cytoplasmic membrane potential gradient [4]. It was discovered that, at concentrations much below their minimum inhibitory concentrations, some peptides only partially collapse the membrane potential of Escherichia coli, whereas other peptides totally depolarize the cytoplasmic membrane at their MICs. The slow-acting human lacto ferrin peptides cause cell lysis, membrane integrity collapse, and membrane potential collapse. Peptides' dual hydrophobic and cationic properties are crucial for the peptide's initial interaction with the bacterial membrane [5,6].

**Antimicrobial Lipids Isolated from fishes**

Antimicrobial lipids, found in natural products like milk, are defence effectors of innate immunity and can kill many pathogens. Free fatty acids (FFAs) are a prominent source of antimicrobial compounds, with their spectra of action and potencies influenced by the degree of saturation, length of carbon chain, and orientation of double bonds. FFAs with cis-orientated carbon-carbon double bonds have greater antimicrobial activities compared to those with trans-orientated bonds [7,8].

Fish by-products can be converted into oil, with omega-3 fatty acids being predominantly found in marine animals. These fatty acids are readily digested for energy production and have various bioactivities. EPA and DHA, found in fish oil, exhibit anti-inflammatory action and increase survival for people with autoimmune diseases. Fish oil treatments have been shown to relieve patients suffering from rheumatoid arthritis, Crohn's disease, and kidney diseases. Omega-3 fatty acids also play a vital role in brain development and reproductive system functions, potentially preventing mental health problems [9].

**Mechanisms of antibacterial activity**

FFAs, or free fatty acids, have antibacterial properties influenced by their structure and shape. The –OH group of the carboxyl group is crucial for FFAs' antibacterial activity, as methylated FFAs often have reduced or no activity. Medium- and long-chain unsaturated FFAs tend to be more active against Gram positive bacteria than Gram-negatives [10].

FFAs exert their antibacterial activities through their amphipathic structures, which allow them to interact with the cell membrane, creating transient or permanent pores of variable size. At higher concentrations, detergents like FFAs can solubilize the membrane, releasing various membrane proteins or larger sections of the lipid bilayer. The key membrane-located process affected by FFAs is the production of energy caused by interference with the electron transport chain and the disruption of oxidative phosphorylation. Other processes that may contribute to bacterial growth inhibition or death

include cell lysis, inhibition of enzyme activity, impairment of nutrient uptake, and the generation of toxic peroxidation and auto-oxidation products [11].
The inner membrane of Gram-positive and Gram-negative bacteria is an important site for energy production, and FFAs can disrupt this process by affecting the electron transport chain and reducing ATP production. Cell lysis can occur due to increased fluidity and permeability of the bacterial inner membrane, leading to growth inhibition or even death. Inhibition of enzymes in the membrane or cytosol that are crucial for bacterial survival and growth could account for some of the antibacterial effects of FFAs [12].
FFAs can also inhibit the ability of bacteria to take up nutrients, such as amino acids, effectively starving them of necessary nutrients. Secondary degradation products of FFAs are responsible for their antibacterial activities, such as peroxidation and auto-oxidation [13].

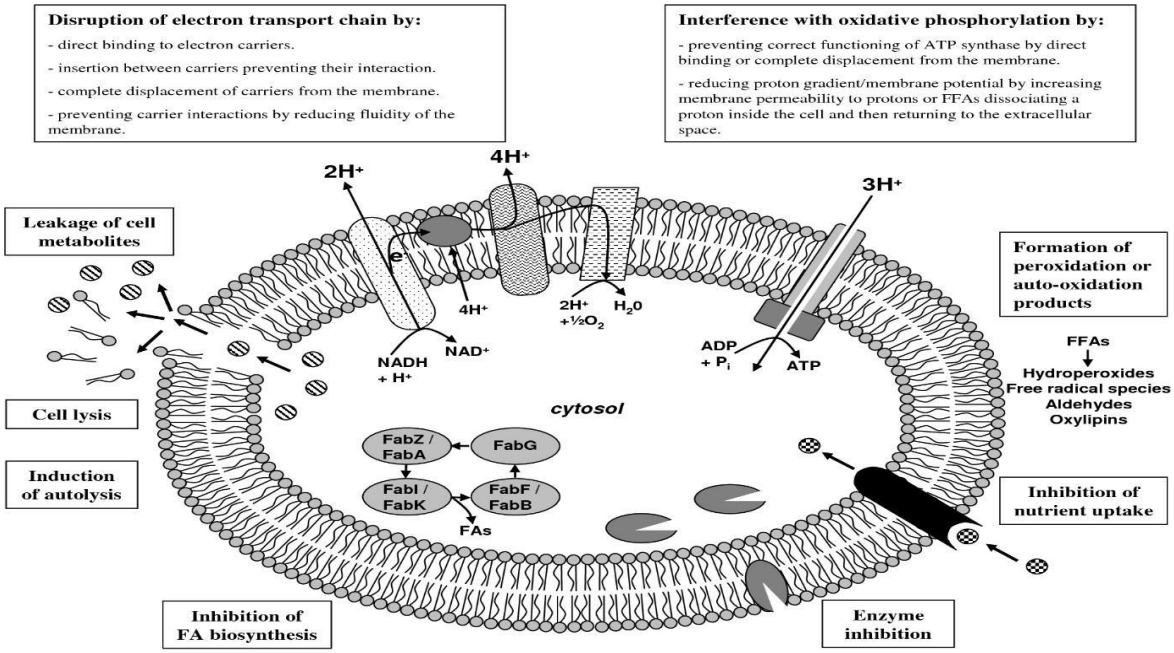


**Figure : 2 Schematic representation of possible cell targets and mechanisms of antibacterial activity of free fatty acids (FFAs). They may affect bacterial energy production by disrupting the electron transport chain and/or interfering with oxidative phosphorylation. FFAs can cause leakage of cell metabolites from the cell, complete cell lysis and autolysis. Membrane and cytosolic enzymes, including those required for fatty acid biosynthesis, can be inhibited by FFAs. They can impair active nutrient uptake by acting directly on the transport protein or as an indirect result of the cell's inability to produce ATP. Peroxidation and auto-oxidation products of FFAs may also have deleterious effects on the bacterial cell and play a role in cell killing. For clarity, only the bacterial inner cell membrane is shown (Adapted from Andrew et al., 2010).**

**SDS-PAGE**
SDS-PAGE is a widely used laboratory technique for separating proteins based on their molecular weight. It involves the use of a polyacrylamide gel matrix and the detergent sodium dodecyl sulphate (SDS), which denatures proteins and gives them a uniform negative charge proportional to their length. The process involves sample preparation, gel preparation, electrophoresis, staining, and visualization. Proteins are mixed with SDS and heated, denatured, and a reducing agent is added to break disulfide bonds [14].
The gel is cast between two glass plates, with small pores acting as a sieve during electrophoresis. The protein samples are loaded into wells at the top of the gel, and an electric field is applied. Larger proteins encounter more resistance and move more slowly through the gel, while smaller proteins move faster [15].

**Protein separation by SDS-PAGE**
Proteins are visualized using staining methods like Coomassie Brilliant Blue or silver staining, which bind to the proteins and reveal their positions as bands in the gel. Alternatively, Western blotting can be used for detection [16].
Protein separation in SDS-PAGE is primarily based on molecular weight, as denaturing conditions eliminate the influence of the protein's shape or charge. The distance a protein migrates in the gel is inversely proportional to its size, allowing researchers to estimate molecular weights based on comparison with standard markers of known sizes [17].

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## METHODOLOGY

### Screening of Antimicrobial Activity

A 5 different concentrations (200, 400, 600, 800, and 1000µg/ml) of the lyophilised protein extracts from the spines, shell and gonads of *Stomopneustes variolaris* were used to evaluate the antimicrobial activity by well diffusion method [18]. The antibacterial activity was assessed by using gram positive bacteria such as *Staphylococcus aureus, Bacillus subtilis*, *Micrococcus luteus* and gram negative bacteria such as *E.coli, Salmonella typhi, Pseudomonas aeruginosa* [19]. For antibacterial activity streptomycin was used as positive control. The antifungal activity was assessed by using fungi such as *Candida albicans, Aspergillus niger,* and *Saccharomyces cerevisiae.* For antifungal activity fluconazole was used as positive control. Agar medium was prepared and pour plate method was performed. 0.5 mm Wells were prepared [20]. Wells were loaded with erythromycin, Elution buffers and 1 mg concentrations of the isolated skin protein extracts from the Chelonodon patoca and Acanthurus mata gels. Plates were sealed with parafilm and incubated at 370C for 24hrs. After incubation, zones of inhibition for extracts were measured in millimeters using vernier calipers[21].

### Statistical Analysis

The results were expressed as mean ± standard deviation of three individual replicates and the data was assessed by one-way analysis of variance (ANOVA) and subjected to regression-correlation analysis by using SPSS 10.0 software. The 'P' value less than 0.05 was considered a significant difference [22-26].

### Protein Extraction

Ferreira et al., (2002) method was used to extract the total protein with slight modifications. 5 gm gonadal of *Stomopneustes variolaris* was weighted and homogenised with pre-frozen mortar and pestle. The sample buffer added with the ratio of sample weight to sample buffer is 3:1. Approximately, 330 ml of sample buffer consisted 0.5 M Tris–HCl (pH 6.8), 0.04% (w/v) SDS, 0.03% (w/v) Dithiothreitol (DTT) and 0.04% (v/v) 2-mercaptoethanol was added to 1 g of sample. Then the homogenates were centrifuged at 14000 rpm for 20 min at 4ºC. The supernatant was collected and stored at -20oC for further protein research analysis [27-29].

### Estimation of Total Protein

The total protein concentration was estimated in the gonads of *Stomopneustes variolaris* by using Lowry et al., (1951) method. To 1 ml of test sample, 4 ml of freshly prepared alkaline solution (prepared by mixing 50 ml of 2% $Na_2CO_3$ in 0.1 N NaOH and 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate) was added at room temperature and kept undisturbed for 10 min. 0.5 ml of Folin-Ciocalteu reagent was added subsequently, to the tubes and after half an hour, the OD of each was measured at 750 nm using a spectrophotometer against the blank (without protein sample). The total protein content in the sample was calculated by referring the ODs of the test sample with the standard curve of BSA. The protein content was expressed in mg per gm fresh weight [30-32].


**Figure : 3 Protein extraction and estimation**

### Preliminary Screening of Antimicrobial Activity with crude protein

The antimicrobial activity was done by the 4 different concentrations such as 25, 50, 75 and 100mg/ml of crude protein extract of *Stomopneustes variolaris* gonads. Antimicrobial activity was done by a well diffusion method. *Klebsiella pneumoniae,* Bacillus licheniformis, E.coli, *Streptococcus pyrogenes, Sphingomonas paucimobilis, Aspergillus flavus* and *Penicillium notatum* were used as test organisms. 100 mg/ml Erythromycin and homogenization buffers were used as positive and negative control. Agar medium was prepared and pour plate method was performed. 0.5 mm Wells were prepared. Wells were loaded with erythromycin, homogenization buffers and different concentrations of the crude protein

extracts from the *Stomopneustes variolaris* gonads. Plates were sealed with parafilm and incubated at 370C for 24hrs. After incubation, zones of inhibition for extracts were measured in millimeters using vernier calipers [33].

**Protein separation by SDS PAGE**

The gonadal tissue of *Stomopneustes variolari* was weighted and homogenized, separately with pre-frozen mortar and pestle. The sample buffer added with the ratio of sample weight to sample buffer is 3:1. Approximately, 330 ml of sample buffer consisted 0.5 M Tris–HCl (pH 6.8), 20.2% (v/v) glycerol, 0.0001% (w/v) (bromophenol blue), 0.04% (w/v) SDS, 0.03% (w/v) Dithiothreitol (DTT) and 0.04% (v/v) 2-mercaptoethanol was added to 1 g of sample. The supernatant of sample was collected and transferred to a new tube after centrifugation at 10,000 × g for 2 min. The supernatant was then boiled at 95◦C for 5 min. The boiled samples were kept in ice before loading into the gel. A 12% resolving gel was prepared according to Laemmli, (1970) containing 1.5 M Tris–HCl (pH 8.8), 20% SDS, 10% ammonium persulphate and TEMED. The stacking gel (5%) was made using 0.5 M Tris–HCl (pH 6.8), 20% SDS, 10% ammonium persulphate and TEMED. The electrophoresis was accomplished at 100 V for 1 h using Vertical Electrophoresis Unit with running buffer consisted 25 mM Tris base, 192 mM glycine and 3 mM SDS. The gel was stained with 0.5% Coomassie Brilliant Blue R-250 in 45% (v/v) methanol, 10% (v/v) acetic acid for one hour and destained for 12 h in 20% (v/v) methanol and 10% (v/v) acetic acid. The gel was washed with distilled water until the background was clear prior to view. Finally, the gel was viewed and photographed with Geldoc.

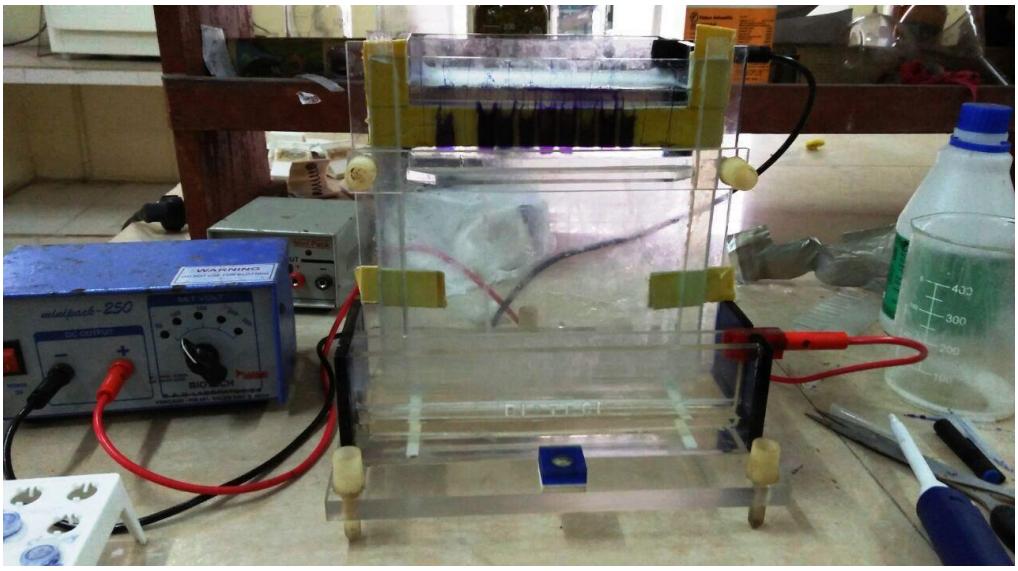


**Figure : 4 SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)**

**Secondary Screening of Antimicrobial Activity with Protein Gel Bands**

After the Gel-electrophoretic procedure, the low molecular weight gel bands from the *Stomopneustes variolaris* gonadal tissue were cut into pieces from the gel and the protein was eluted from the gel matrix with the gel elution buffer pH-7.5. After immersing the gel pieces in an elution buffer, the sample is centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant containing the isolated protein/peptide was further analyzed for antimicrobial activity. Antimicrobial activity was done by a well diffusion method. *Klebsiella pneumoniae*, *Bacillussubtilis*, E.coli, *Streptococcus pyrogenes*, Sphingomonas paucimobilis, Aspergillus flavus and Penicillium notatum were used as test organisms. 100 mg/ml Erythromycin and homogenization buffers were used as positive and negative control. Agar medium was prepared and pour plate method was performed. 0.5 mm Wells were prepared. Wells were loaded with erythromycin, Elution buffers and eluted protein extracts from the *Stomopneustes variolaris* gonadal tissue gels. Plates were sealed with parafilm and incubated at 370C for 24hrs. After incubation, zones of inhibition for extracts were measured in millimeters using vernier calipers.

**RESULT AND DISCUSSION**

**Antimicrobial Activity**

A 5 different concentrations (200, 400, 600, 800, and 1000µg/ml) of the lyophilised protein extracts from the spines, shell and gonads of Stomopneustes Variolaris Were used to evaluate the antimicrobial activity by well diffusion method. The antibacterial activity was assessed by using gram positive bacteria such as Staphylococcus aureus, *Bacillus subtilis*, *Micrococcus luteus* and gram-negative bacteria such as E.coli, *Salmonella typhi*, *Pseudomonas aeruginosa*. For antibacterial activity streptomycin was used as positive control. The antifungal activity was assessed by using fungi such

as *Candida albicans*, *Aspergillus niger*, and *Saccharomyces cerevisiae*. For antifungal activity fluconazole was used as positive control. All the results expressed as mean value ± standard deviation of three replicates.
The Stomopneustesvariolarigonad protein extract showed maximum antimicrobial activity at 1mg concentration. Among all the tested gram positive, gram negative bacteria, and fungi the gonad protein extract showed maximum antimicrobial activity against Staphylococcus aureus(18 mm), Pseudomonas aeruginosa (17 mm), and Aspergillus Niger (15 mm)respectively at 1 mg/ml concentration. The results were shown in Table.1. The antimicrobial activity of *Stomopneustes Variolaris* Gonad protein extract shows significant increase with increasing extract concentration.The extracts show strong positive correlation with increasing extract concentration.The gonadal protein extracts with increasing concentration show a very strong positive correlations such as r = 0.973, r = 0.857, r = 0.962, r = 0.944, r = 0.857, r = 0.984, r = 0.938, r = 0.919 and r = 0 against to the Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, E.coli, Salmonella typhi, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger, and Saccharomyces cerevisiaerespectively. The results were shown in Table.2.

**Table: 1 Antimicrobial activity of Stomopneustes variolaris Gonadal protein extracts with increasing concentration.**

| Name of the Organism | | Diameter of the Inhibition Zone (mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Stomopneustesvariolari*Gonadal Protein (mg/ml) | | | | | Positive Control | Negative Control |
| | | **0.2** | **0.4** | **0.6** | **0.8** | **1** | | |
| **Gram Positive Bacteria** | *Staphylococcus aureus* | 8 | 12 | 15 | 16 | 18 | 33 | -- |
| | *Bacillus subtilis* | 0 | 9 | 10 | 12 | 12 | 32 | -- |
| | *Micrococcus luteus* | 8 | 9 | 9 | 11 | 12 | 26 | -- |
| **Gram negative Bacteria** | *Escherichia coli* | 8 | 10 | 13 | 13 | 14 | 33 | -- |
| | *Salmonella typhi* | 0 | 9 | 10 | 12 | 12 | 32 | -- |
| | *Pseudomonas aeruginosa* | 11 | 13 | 15 | 16 | 17 | 26 | -- |
| **Fungi** | *Candida albicans* | 0 | 0 | 8 | 12 | 12 | 25 | -- |
| | *Aspergillus niger* | 0 | 0 | 12 | 14 | 15 | 18 | -- |
| | *Saccharomyces cerevisiae* | 0 | 0 | 0 | 0 | 0 | 22 | -- |

**Table: 2 Correlation of Antimicrobial activity of *Stomopneustesvariolari*Gonadalprotein extracts with increasing concentration**

| | ***Ex.Conc*** | ***S.aureus*** | ***B.subtilis*** | ***M.luteus*** | ***E.coli*** | ***S.typhi*** | ***P.aeruginosa*** | ***C.albicans*** | ***A.niger*** | ***S.cerevisiae*** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Ex.Conc*** | 1 | | | | | | | | | |
| ***S.aureus*** | 0.973 | 1 | | | | | | | | |
| ***B.subtilis*** | 0.857 | 0.934 | 1 | | | | | | | |
| ***M.luteus*** | 0.962 | 0.889 | 0.782 | 1 | | | | | | |
| ***E.coli*** | 0.944 | 0.986 | 0.904 | 0.824 | 1 | | | | | |
| ***S.typhi*** | 0.857 | 0.934 | 1 | 0.782 | 0.904 | 1 | | | | |
| ***P.aeruginosa*** | 0.984 | 0.995 | 0.913 | 0.909 | 0.984 | 0.913 | 1 | | | |
| ***C.albicans*** | 0.938 | 0.913 | 0.767 | 0.862 | 0.932 | 0.767 | 0.944 | 1 | | |
| ***A.niger*** | 0.919 | 0.917 | 0.765 | 0.808 | 0.953 | 0.765 | 0.941 | 0.989 | 1 | |
| ***S.cerevisiae*** | -- | -- | -- | -- | -- | -- | -- | -- | -- | 1 |

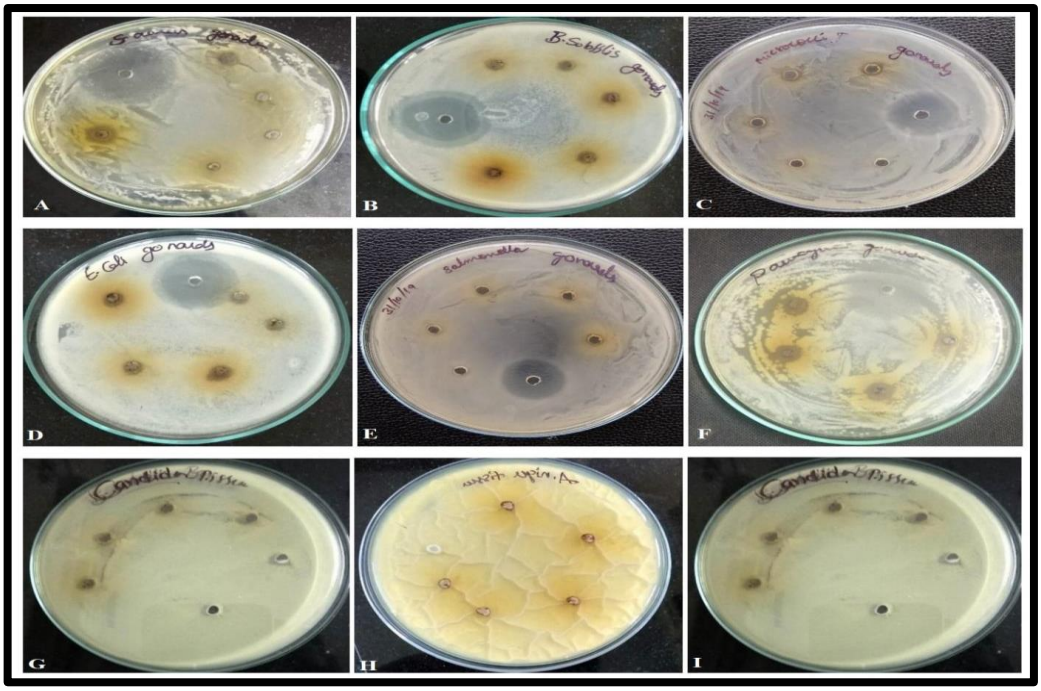

**Figure : 5. Antimicrobial activity of *Stomopneustesvariolari*Gonadalprotein extracts with increasing concentration. A) Inhibitory zones of *Staphylococcus aureus*,B) Inhibitory zones of *Bacillus subtilis*,C) Inhibitory zones of *Micrococcus luteus*,D) Inhibitory zones of *E.coli*,E)Inhibitory zones of *Salmonella typhi*, F)Inhibitory zonesof *Pseudomonas aeruginosa*, G)Inhibitory zonesof *Candida albicans*, H)Inhibitory zones of *Aspergillus niger*, I)Inhibitory zones of *Saccharomyces cerevisiae*.**

The *Stomopneustes Variolaris* shell protein extract showed maximum antimicrobial activity at 1mg concentration. Among all the tested gram positive, gram negative bacteria, and fungi the shell protein extract showed maximum antimicrobial activity against *Staphylococcus aureus* (10mm), *Pseudomonas aeruginosa* (11 mm), and *Candida albicans* (11mm)respectively at 1 mg/ml concentration. The results were shown in Table.3. The antimicrobial activity of *Stomopneustes Variolaris shell Protein* extract shows significant increase with increasing extract concentration. The extracts show strong positive correlation with increasing extract concentration.The shell protein extracts with increasing concentration show a very strong positive correlations such as r = 0.970, r = 0.882, r = 0.812, r = 0.823, r = 0.882, r = 0.919, r = 0, r = 0 and r = 0 against to the *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *E.coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, and *Saccharomyces cerevisiae* respectively. The results were shown in Table.4.

**Table: 3. Antimicrobial activity of *Stomopneustes variolaris Shell protein* extracts with increasing concentration.**

| Name of the Organism | | Diameter of the Inhibition Zone (mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Stomopneustesvariolari*Gonadal Protein (mg/ml) | | | | | Positive Control | Negative Control |
| | | **0.2** | **0.4** | **0.6** | **0.8** | **1** | | |
| **Gram Positive Bacteria** | *Staphylococcus aureus* | 0 | 8 | 8 | 10 | 10 | 33 | -- |
| | *Bacillus subtilis* | 0 | 7 | 8 | 9 | 9 | 32 | -- |
| | *Micrococcus luteus* | 0 | 0 | 9 | 8 | 9 | 26 | -- |
| **Gram negative Bacteria** | *Escherichia coli* | 0 | 8 | 8 | 9 | 10 | 33 | -- |
| | *Salmonella typhi* | 0 | 7 | 8 | 9 | 9 | 32 | -- |
| | *Pseudomonas aeruginosa* | 0 | 8 | 8 | 10 | 11 | 26 | -- |
| **Fungi** | *Candida albicans* | 0 | 0 | 8 | 9 | 11 | 25 | -- |
| | *Aspergillus niger* | 0 | 0 | 0 | 8 | 9 | 18 | -- |
| | *Saccharomyces cerevisiae* | 0 | 0 | 0 | 0 | 0 | 22 | -- |

**Table. 4 .Correlation of Antimicrobial activity of *Stomopneustes variolaris Shell protein* extracts with increasing concentration**

| | ***Ex.Conc*** | ***S.aureus*** | ***B.subtilis*** | ***M.luteus*** | ***E.coli*** | ***S.typhi*** | ***P.aeruginosa*** | ***C.albicans*** | ***A.niger*** | ***S.cerevisiae*** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Ex.Conc*** | 1 | | | | | | | | | |
| ***S.aureus*** | 0.970 | 1 | | | | | | | | |
| ***B.subtilis*** | 0.882 | 0.930 | 1 | | | | | | | |
| ***M.luteus*** | 0.812 | 0.665 | 0.519 | 1 | | | | | | |
| ***E. coli*** | 0.823 | 0.679 | 0.532 | 0.999 | 1 | | | | | |
| ***S.typhi*** | 0.882 | 0.930 | 1 | 0.519 | 0.532 | 1 | | | | |
| ***P.aeruginosa*** | 0.919 | 0.928 | 0.730 | 0.721 | 0.734 | 0.730 | 1 | | | |
| ***C.albicans*** | -- | -- | -- | -- | -- | -- | -- | 1 | | |
| ***A.niger*** | -- | -- | -- | -- | -- | -- | -- | -- | 1 | |
| ***S.cerevisiae*** | -- | -- | -- | -- | -- | -- | -- | -- | -- | 1 |

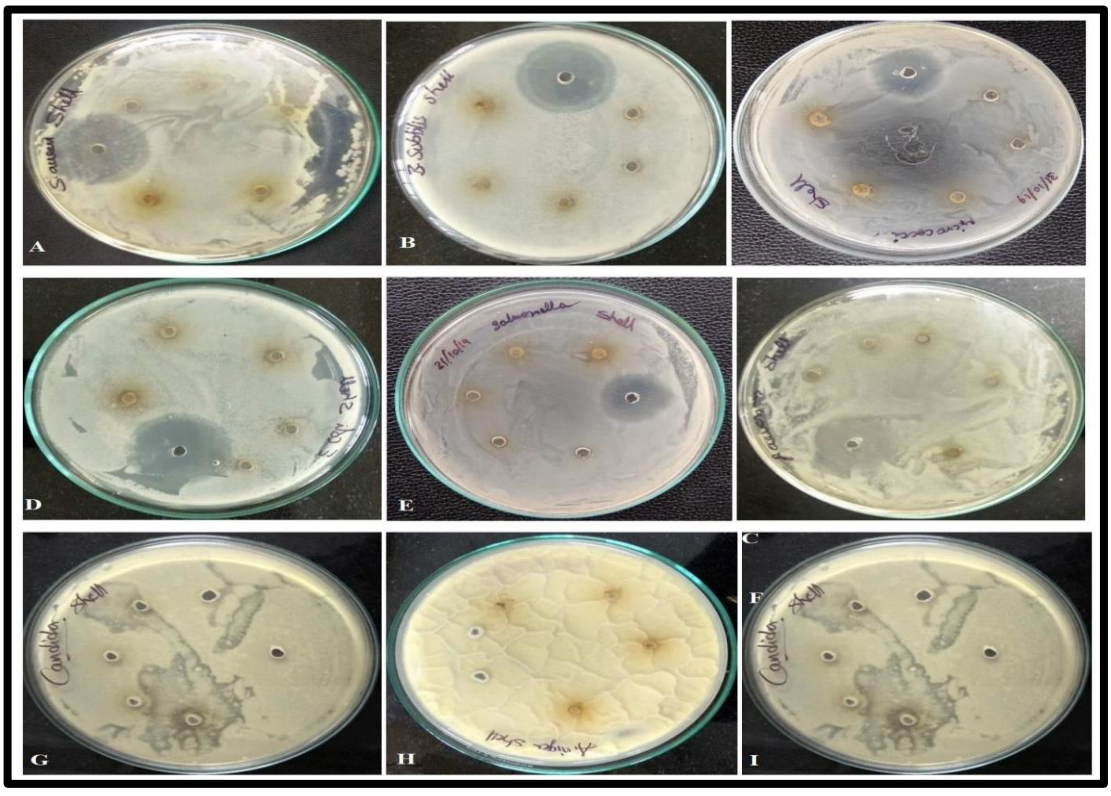


**Figure: 6. Antimicrobial activity of *Stomopneustes variolaris Shell protein* extracts with increasing concentration. A) Inhibitory zones of *Staphylococcus aureus*, B) Inhibitory zones of *Bacillus subtilis*, C) Inhibitory zones of *Micrococcus luteus*, D) Inhibitory zones of *E.coli*, E) Inhibitory zones of *Salmonella typhi*, F) Inhibitory zones of *Pseudomonas aeruginosa*, G) Inhibitory zones of *Candida albicans*, H) Inhibitory zones of *Aspergillus niger*, I) Inhibitory zones of *Saccharomyces cerevisiae.***

The *Stomopneustes Variolaris spine protein* extract showed maximum antimicrobial activity at 1mg concentration. Among all the tested gram positive, gram-negative bacteria, the spine protein extract showed maximum antimicrobial activity against *Staphylococcus aureus* (13mm), *Pseudomonas aeruginosa* (10 mm) respectively and no activity was found against fungus at 1 mg/ml concentration. The results were shown in Table.5. The antimicrobial activity of *Stomopneustes variolaris* spine protein extract shows significant increase with increasing extract concentration. The extracts show strong positive correlation with increasing extract concentration.The spine protein extracts with increasing concentration show a very strong positive correlations such as r = 0.971, r = 0.882, r = 0.812, r = 0.823, r = 0.882, r = 0.919, r = 0, r = 0 and r =

0 against to the *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, E.coli, Salmonella typhi, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger*, and *Saccharomyces cerevisiae* respectively. The results were shown in Table.6.

**Table:5. Antimicrobial activity of *Stomopneustes variolaris* Spine protein extracts with increasing concentration**.

| Name of the Organism | | Diameter of the Inhibition Zone (mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Stomopneustesvariolari*Gonadal Protein (mg/ml) | | | | | Positive Control | Negative Control |
| | | **0.2** | **0.4** | **0.6** | **0.8** | **1** | | |
| **Gram Positive Bacteria** | *Staphylococcus aureus* | 8 | 8 | 10 | 12 | 13 | 33 | -- |
| | *Bacillus subtilis* | 0 | 0 | 0 | 7 | 8 | 32 | -- |
| | *Micrococcus luteus* | 0 | 8 | 9 | 9 | 10 | 26 | -- |
| **Gram negative Bacteria** | *Escherichia coli* | 0 | 7 | 8 | 8 | 9 | 33 | -- |
| | *Salmonella typhi* | 0 | 0 | 0 | 7 | 8 | 32 | -- |
| | *Pseudomonas aeruginosa* | 0 | 0 | 8 | 8 | 10 | 26 | -- |
| **Fungi** | *Candida albicans* | 0 | 0 | 0 | 0 | 0 | 25 | -- |
| | *Aspergillus niger* | 0 | 0 | 0 | 0 | 0 | 18 | -- |
| | *Saccharomyces cerevisiae* | 0 | 0 | 0 | 0 | 0 | 22 | -- |

**Table.6. Correlation of Antimicrobial activity of *Stomopneustes variolaris* Spine protein extracts with increasing concentration.**

| | ***Ex.Conc*** | ***S.aureus*** | ***B.subtilis*** | ***M.luteus*** | ***E.coli*** | ***S.typhi*** | ***P.aeruginosa*** | ***C.albicans*** | ***A.niger*** | ***S.cerevisiae*** |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Ex.Conc*** | 1 | | | | | | | | | |
| ***S.aureus*** | 0.9707 | 1 | | | | | | | | |
| ***B.subtilis*** | 0.8820 | 0.9306 | 1 | | | | | | | |
| ***M.luteus*** | 0.8125 | 0.6653 | 0.5193 | 1 | | | | | | |
| ***E.coli*** | 0.8237 | 0.6793 | 0.5320 | 0.9997 | 1 | | | | | |
| ***S.typhi*** | 0.8820 | 0.9306 | 1 | 0.5193 | 0.5320 | 1 | | | | |
| ***P.aeruginosa*** | 0.9191 | 0.9286 | 0.7301 | 0.7214 | 0.7343 | 0.7301 | 1 | | | |
| ***C.albicans*** | -- | -- | -- | -- | -- | -- | -- | 1 | | |
| ***A.niger*** | -- | -- | -- | -- | -- | -- | -- | -- | 1 | |
| ***S.cerevisiae*** | -- | -- | -- | -- | -- | -- | -- | -- | -- | 1 |

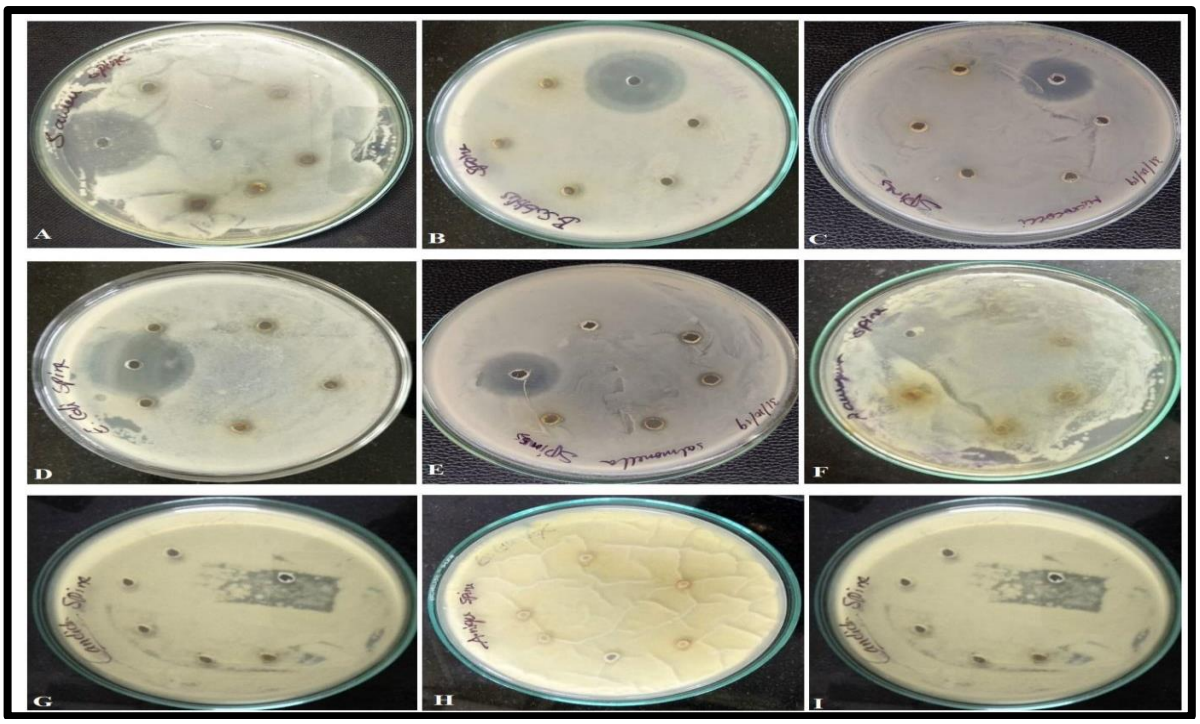


**Fig: 7. Antimicrobial activity of *Stomopneustesvariolari*Spineprotein extracts with increasing concentration. A) Inhibitory zones of *Staphylococcus aureus*, B) Inhibitory zones of *Bacillus subtilis*, C) Inhibitory zones of *Micrococcus luteus*, D) Inhibitory zones of *E.coli*, E) Inhibitory zones of *Salmonella typhi*, F) Inhibitory zones of *Pseudomonas aeruginosa*, G) Inhibitory zones of *Candida albicans*, H) Inhibitory zones of *Aspergillus niger*, I) Inhibitory zones of *Saccharomyces cerevisiae.***

In the present investigation the tested protein extracts from *Stomopneustes variolaris* gonads, shell and spine shows better antimicrobial activity against bacterial species than fungal species.
Lauth*et al.*, (2002) found that antimicrobial proteins interact directly with bacteria and kill them.Squalamine, a cationic steroidal antibiotic isolated from the dogfish shark has been shown to exert broad-spectrum antimicrobial action against Gram-negative and positive bacteria as well as fungi and protozoa (Moore *et al.*, 1993; Wehrli*et al.*, 1993).

**Protein Estimation**

The final results were expressed from the three independent experiments as Mean ± Standard deviation. The protein content in the skin of *Chelonodon Patoca* and *Acanthurusmata*shows significant variation. The concentration of protein in the skin tissue of *Chelonodon Patoca* and *Acanthurusmata*were reported as 12±3 and 15±1 mg/gmFW respectively. The results were shown in Figure : 9.

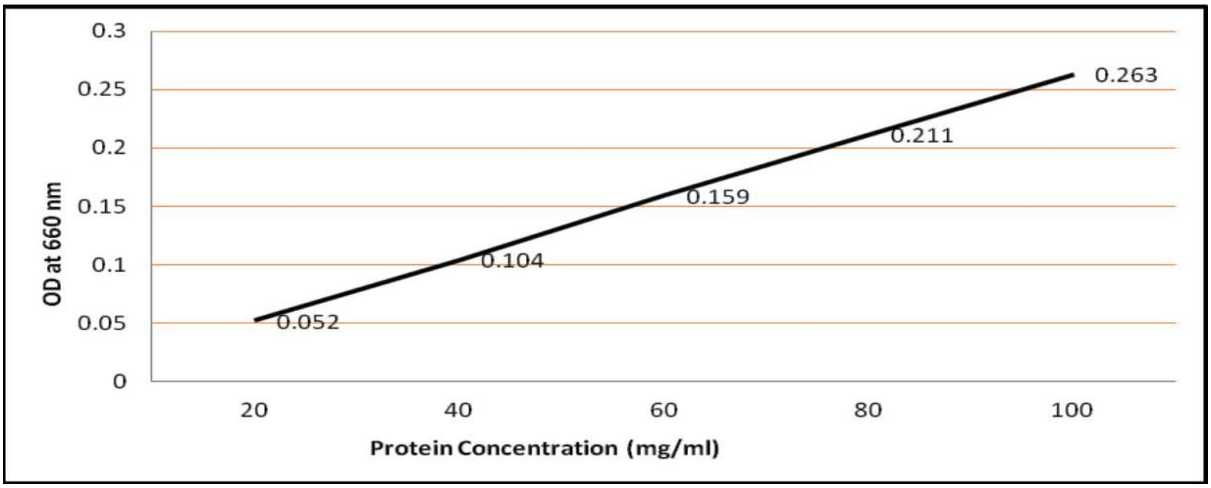


**Figure : 8 Protein standard graph.**

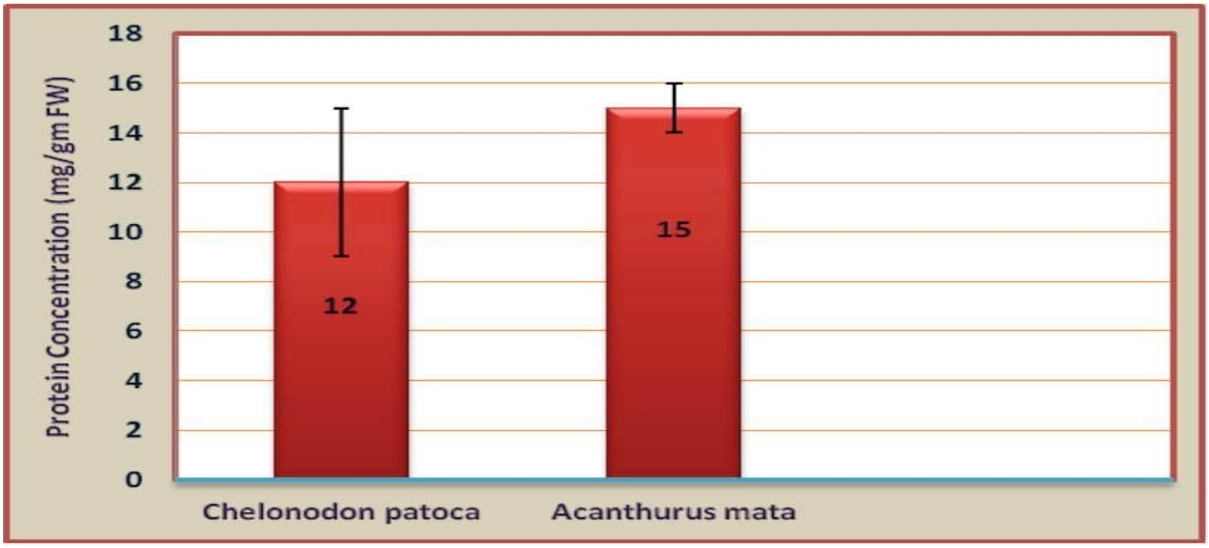


**Figure : 9 Total skin protein content of *Chelonodonpatoca* and *Acanthurusmata*.**

Pharmacognostic evaluation of crude extracts plays a very important role in identification of novel bioactive compounds. Various kinds of marine organisms, from different tidal habitats are the rich source of pharmaceuticals. *Chelonodon Patoca* and *Acanthurus Mata are* marine fishes screened for bioactive materials. The knowledge of bioactive potential of any organisms is extremely important, because the medicinal value is reflected in its biochemical contents (Nagabhushanam and Mane, 1978). Protein is an important biochemical macronutrient and plays an important role in determining the texture and quality of an organism.

In human nutrition, fish is the major source of food which provides the highest protein and lipid content. Among the biomolecular composition, the fish contain 20-30% of protein (Love,1980). The present results agree with the earlier works such as Hossain, *et al.,* (2011) reported that the proper structure and functions of all organisms including fish requires a significant amount of protein. The protein content of skin in the present study is similar to muscle protein content of some popularly cultured marine fish such as silver pomfret, sea bream and grouper which ranged between 16.25 and 18.83%**.** According to Akinneye, (2010) The significant variation in the skin protein content of *Chelonodonpatoca* and *Acanthurusmata* mostly associated with the periodic and biological changes such as geographical position, food resources, species, size, age, sex, muscle volume, and environmental conditions like water salinity, temperature and other pollutants.

The current results collinear with earlier works likeDhaneesh*et al.,* (2012) who reported that the protein contents were high in *Thunnus Albacares* (13.69%), *Parupeneus Bifasciatus* (6.12%), *Hyporhamphusdussumieri* (6.97%) and *T. albacares* (1.65%) respectively. Nurnadia, (2011) reported that the highest protein content in Moonfish was 6.89+2.76%. Protein content in fishes normally present in the range of 17 to 18%, but in rare cases the protein content has been observed as lower (13-5%) or higher (20-1%) than the normal (Natarajan and Sreenivasan, 1961). Jitender kumar *et al.,* (2012) has reported that the protein content of several fishes such as Catla, Rohu, Magur, and Pangas as 10.11%, 9.53%, 14.87%, and 13.6% correspondingly. Eswar *et al.,* (2014) has been reported that the protein concentrations in puffer fishes such as *Lagocephalus Lunaris And Lagocephalus Inermis*. Kumaran et al., (2012) reported that the protein content of *Mugil cephalus* was 17.56%.

**Protein profiling by SDS PAGE**

SDS PAGE provides an excellent method to separation and quantitative, qualitative analysis of the expressed proteins based on their molecular weight.The expressed proteome of *Chelonodon Patoca* and *Acanthurus Meta molecular* weights ranges between 1 – 200 kilodaltons. Analysis of *Chelonodonpatoca*proteome revealed that there were more proteins with low molecular weight. Three proteins which are having molecular weights of 200, 116 and 10 KD were darkly stained, which indicates they were quantitatively highly expressed proteins. Low molecular weight proteins were situated after the 45 KDmarkers. Among the prominently expressed proteins the very low molecular weight 10KD protein from the gel of *Chelonodonpatoca*was selected for the antimicrobial activity and MALDI-TOF analysis for identification of the protein. Analysis of *Acanthurusmata*proteome also revealed that there were more proteins that are low molecular weight. Four proteins which are having molecular weights of 134, 78, 57 and 9 KD were darkly stained, which indicates they were quantitatively highly expressed proteins. Low molecular weight proteins were situated after the 45 KD markers. Among the prominently expressed proteins the very low molecular weight 9 KD proteins from the gel of *Acanthurusmata*was selected for the antimicrobial activity and MALDI-TOF analysis for identification of the protein.The identification of low

molecular weight antimicrobial proteins from *Chelonodonpatoca* and *Acanthurusmata* provides new insights in the field of pharmaceutical biotechnology.

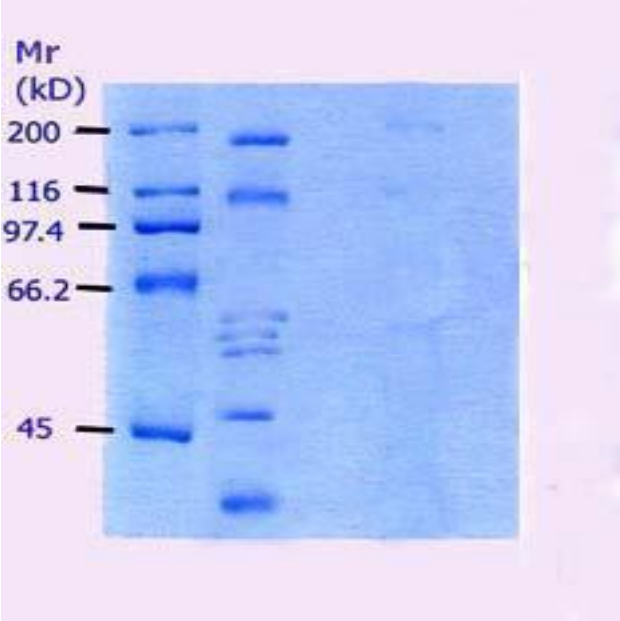


**Figure : 10. Protein profiles of *Chelonodonpatoca* and *Acanthurusmata*by SDS polyacrylamide gel.**

Several types of antimicrobial peptides (AMPs) can be found in fish which contain a short chain of positively charged amino acid residues and these peptides are involved in defense mechanisms. Zhang *et al.,* (2009) has been proposed that the antimicrobial peptides play a vital role in innate immunity by interacting and killing of bacteria directly. Hence, this kind of antimicrobial peptide can be used instead of conventional antibiotics because the antimicrobial peptide kills bacteria without developing antibiotic resistant mechanisms (Shahidi and Zhong, 2008).
Several researchers isolated the antimicrobial peptides from fishes. Two antimicrobial proteins were isolated by Lemaitre *et al.,* (1996) which are having 27 and 31 KDa molecular weight and are involved in the antimicrobial activity of carp skin mucus. Cole *et al.,* 1997; Douglas *et al.,* 2001, 2003a, 2003b have been isolated antimicrobial peptides from winter flounder, *Pleuronectes americanus*, *America placia*, *Hippoglossoides platessoides* and *Hippoglossus hippoglossus*. Zhang et al., (2008) have isolated the antimicrobial fraction from the skin of *Epinephelusfario*. Bergsson et al., (2005) has been found that the epithelium of fish skin and other mucosal surfaces are rich in antimicrobial proteins.

**Antimicrobial Activity of Biopeptides**
The extracted peptide fractions from the SDS polyacrylamide gels of *Chelonodon Patoca* and *Acanthurusmata* using the optimized conditions for their antimicrobial activity. Chelonodon Patoca and *Acanthurus mata* biopeptide fractions with molecular weight cutoffs10 KDa and 9KDa were prepared. The prepared skin protein extracts were from the test for antimicrobial activity against four bacterial species viz.*Pseudomonas aeruginosa*, *Bacillus subtilis*, *E.coli*, *Klebsiella pneumonia* and two fungal species viz. *Aspergillus flavus and Penicillium notatum*. The antimicrobial activity of 10KDa and 9KDa biopeptide from the *Chelonodonpatoca*and*Acanthurusmata* skin is given in Table 7.
The 10KDa biopeptide of *Chelonodon Patoca Showed* maximum antimicrobial activity against *Bacillus subtilis* (20mm) followed by *E.coli* (19mm), *Klebsiella pneumonia* (19mm), *Aspergillus flavus* (17mm), *Pseudomonas aeruginosa* (16mm) and *Penicillium notatum* (15mm). The 9KDa biopeptide of *Acanthurusmata*showed maximum antimicrobial activity against *Klebsiella pneumonia* (29mm) followed by *E.coli* (18mm), *Aspergillus flavus* (18mm), *Klebsiella pneumonia* (19mm), *Bacillus subtilis* (20mm), *Pseudomonas aeruginosa* (15mm) and *Penicillium notatum* (15mm).

**Table: 7 Antimicrobial activity from the skin protein extracts of *Chelonodon Patoca* and *Acanthurusmata.***

| Name of the Organism | Diameter of the Zone (mm) | | Positive Control (Erythromycin) | Negative Control (Elution Buffers) |
|---|---|---|---|---|
| | *Chelonodon patoca* | *Acanthurusmata* | | |
| *Pseudomonas aeruginosa* | 16 | 15 | 14 | - |
| *Bacillus subtilis* | 20 | 16 | 14 | - |
| *Escherichia coli* | 19 | 18 | 14 | - |

| | | | | |
|---|---|---|---|---|
| *Klebsiella pneumonia* | 19 | 29 | 14 | - |
| *Aspergillus flavus* | 17 | 18 | 14 | - |
| *Penicillium notatum* | 15 | 15 | 14 | - |

Collagen and gelatin are the main constituents in the fish skin which are composed with repeated units of amino acid sequence gly-pro-ala and these amino acid sequences are responsible for the bioactivities such as antimicrobial activity. Byun and Kim, (2001) and Mendiset *et al.,* (2005) reported that the skin peptides which are isolated by enzymatic hydrolysis show better bioactivity than the peptides isolated from the fish muscle. The present results collinear with earlier findings such as Gomez et al., (2010) have been reported that the 1-10 KDa peptide fractions of tuna and squid skin gelatin shows antimicrobial activity. Anbuchezhian *et al.,* (2011) observed significant antimicrobial activity with the peptides isolated from the estuarine catfish *Mystus Gulio* epidermal mucous. Rameshkumar *et al.,* (2009) reported that the bioactive peptides extracted from the *Thalamita Crenata* exhibit good antimicrobial activity against human bacterial pathogens. Lauthet *et al.,* (2002) reported that the antimicrobial peptides kill bacteria by interacting directly with the bacterial cells. A cationic steroidal antibiotic, known as squalamine which is isolated from the dogfish shark exhibit broad-spectrum antimicrobial activity against fungi, protozoa and, Gram +ve bacteria and Gram -ve bacteria (Moore *et al.,* 1993; Wehrli *et al.,* 1993). In the present investigation the tested biopeptides showed good antimicrobial activity against bacterial species than fungal species.

## CONCLUSION

The study assessed the antimicrobial activity of lyophilized protein extracts from Stomopneustes variolari spines, shell, and gonads using a well diffusion method. The antibacterial activity was assessed using gram-positive and gram-negative bacteria, with streptomycin as a positive control. Fungi such as Candida albicans, Aspergillus niger, and *Saccharomyces cerevisiae* were also assessed. The *Stomopneustes variolaris* gonad protein extract showed maximum antimicrobial activity at 1mg concentration, while the shell protein extract showed maximum antimicrobial activity against Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans.

The study also investigated the antimicrobial activity of protein extracts from *Stomopneustes variolaris* gonads, specifically the shell and spine protein extracts against bacterial species. The protein content in the skin of Chelonodon Patoca and Acanthurusmata showed significant variation, with concentrations of 12±3 and 15±1 mg/gmFW, respectively. Protein is an important biochemical macronutrient and plays a crucial role in determining the texture and quality of an organism.

Antimicrobial peptides (AMPs) found in fish contain short chains of positively charged amino acid residues that play a vital role in innate immunity by directly killing bacteria. Researchers isolated antimicrobial peptides from various fish species, including carp skin mucus, winter flounder, Pleuronectes americanus, America placia, Hippoglossoides platessoides, and Hippoglossus hippoglossus. The extracted peptide fractions from the SDS polyacrylamide gels of Chelonodon Patoca and Acanthurusmata were tested for antimicrobial activity against four bacterial species and two fungal species.

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