In the past 30-40 years, marine plants and animals have been focused worldwide to define the natural product of the marine environment. Discovery efforts have yielded several bioactive metabolites that have successfully entered the clinical trials. Their large scale production had been developed by pharmaceutical industries (Konig et al., 1999). Not only the natural products but also their derived analogue molecules are currently being used for clinical purposes. Biodiscovery is the extraction and testing of molecules for biological activity. Identification of compounds ensure for further development and research on the molecular basis for the biological activity. With regard to drug discovery and development from marine resources, the researchers had started with a sponge derived nucleosides, spongothymidine and spongoruidine in early 19th century itself (Bergmann and Fenney, 1931). About 14,000 different natural products from marine organisms have been described (Marin Lit, 2003). Hundreds of patents describing new bioactive marine natural product have been filed (Kerr and Kerr, 1999). Approximately 10-15 different marine natural products are currently in clinical trials mostly in the arena of cancer and inflammatory therapeutics diseases (Proksch et al., 2002).

Generally fish consumption is associated with health benefits owing to its high nutritional value (protein, minerals, vitamins and distinctive lipids). Reports from major fishing countries, indicates that fish provides more than 2.6 billion people with at least 20% of their average animal protein intake. Fish can serve as a source of functional materials, such as polyunsaturated fatty acids, polysaccharides, minerals
and vitamins, antioxidants, enzymes and bioactive peptides. Several immunomodulating, antimicrobial, antithrombotic, hypotensive and anticancer peptides had been discovered from fishes in the recent decades.

9.1.2. Antimicrobial Peptides

The antimicrobial peptides (AMPs) play a vital defense mechanism in lower organisms against microbial infections and considered as an important component of the innate immune response of mammals including humans. Currently, AMPs database listed more than 100 natural host defence peptides that exhibits promising antitumor activity (Wang and Wang, 2004; Wang et al., 2009). AMPs were obtained from a wide variety of organisms. These molecules have attracted much research interest because of their biochemical diversity, broad specificity to act against viral, bacterial, fungal pathogens and protozoan parasites.

In the last decade, many species-specific AMPs have been isolated from fish and showed broad spectrum of activity against Gram-positive and Gram-negative bacteria (Lauth et al., 2002; Patrzykat et al., 2003). Rameshkumar et al. (2009b) proved that marine crabs Charybdis lucifera possess an antibacterial peptide in their haemolymph. AMPs were also isolated from Thalamita crenata and it showed promising activity towards human pathogens (Rameshkumar et al., 2009a). Oren and Shai (1996) isolated paradaxin, a 33 amino acid pore forming polypeptide toxin isolated from red sea moses, sole fish and evidenced antibacterial activity and haemolytic activity. Wang et al. (2006) isolated AMPs from the tissue of catfish.

Generally, skin secretions contain a wide variety of polypeptides with antimicrobial properties. Proteases are considered to be antimicrobial proteins which are involved in the regulatory production of antimicrobial peptides. Recently, number of AMPs has been isolated from a wide number of fish species. Among which
pleurocidin from winter flounder (Pleuronectes americanus), cathelicidins from rainbow trout (Oncorhynchus mykiss), defensins from zebrafish (Danio rerio), piscidins from hybrid striped bass (white bass, Morone chrysops, X striped bass, Morone saxatilis), dicentracin from seabass (Dicentrarchus labrax) hepcidin from channel catfish (Ictalurus punctatus) and epinecidin from the grouper (Epinephelus coiodes) are the remarkable ones.

The skin epithelium, serum and mucosal surfaces of fish are rich in AMPs (Bergsson et al., 2005). Earlier reports have confirmed the antimicrobial activity of serum of Atlantic Cod and Coho salmon (Patrzykat et al., 2001; Caipang et al., 2008). Xiaomei et al. (2012) identified and characterised the antimicrobial peptide from catfish Clarias gariepinus. Antimicrobial peptide extends to suppress the tumour cells and wound-healing effects (Zasloff, 2002). They do play an important role in innate immunity and can directly interact with bacteria and causes lethal effect (Lauth et al., 2002). Antimicrobial and antifungal activities of mucus extracts against human and fish pathogens were tested along with ampicillin as control (Uthayakumar et al., 2012).

Cancer is a leading cause of death worldwide representing about one-eighth of all deaths. In 2008, more than 12.7 million people were newly diagnosed with cancer. Furthermore, cancer has also emerged as a major public health problem in developing countries. According to the World Health Organization new cases of cancer impairment will strongly increase with estimated death rates of up to 11 million in the year 2030. Data from population based cancer registries in India shows that the most frequently reported cancer sites in male are lung, oesophagus, stomach and larynx and in female are cervix, breast, ovary and oesophagus (Gajalakshmi et al., 2009). Breast cancer accounts for 19.3% of all cancer cases. Among Indian women breast
Breast cancer is the second most common cancer after cervix cancer. Breast cancer refers to a malignant tumor that has developed from cells in the breast. The age standardized rates vary from 9 to 28.6% per 100,000 women. The present scenario in India reports roughly 100,000 new cases annually and 1 in 26 women are expected to be diagnosed with breast cancer in their lifetime.

Although in recent decades much progress has been achieved in respect of therapies like surgery, chemotherapy, radiation or hormone ablation therapy. They are not successful in more than 50% of cases. Furthermore, for those who survive, the risk of reoccurrence of the disease is a major risk factor. If the tumor progresses or reoccurs, chemotherapy is the standard treatment. However, resistance as well as potential toxicity and the increased side effects of chemotherapeutics, which are mainly due to inadequate specificity for tumor cells that represents a major limitation in this type of therapy. Despite the high number of available drugs, there is a growing need to develop more specific agents to treat cancers, particularly against chemo-resistant tumors. The AMPs revealed cytotoxic activity in cancer cells and are described in terms of their structure and mode of action (Hoskin and Ramamoorthy, 2008). These host defense peptides are characterized by their low molecular weight comprising of about 30 amino acids in general and confers low antigenicity (Iwaski et al., 2009).

Moray eels which are belongs to the order Anguilliformes have toxic proteins in their blood and are usually referred to as ichthyotoxins. The moray eel serum was thought to be haemolytic in nature. Ichthyotoxins may be used for curing kidney inflammation, heart problems and burning sensation of the kidneys with doubt of uremia. This protein can be used to create a new drug for kidney inflammation, cancer, cardiovascular diseases (Vidhya et al., 2013).
Information and literature on isolation and characterization of serum protein is very limited. Therefore, this study was aimed to isolate and characterize the serum protein from Moray eel, *Thyreoidea macrura*. Furthermore the efficiency of characterized protein to act against human bacterial and fungal pathogens as well as the anti cancer potential was investigated.
9.2. MATERIALS AND METHODS

In the present study, live Moray eels were collected from Annan Kovil landing centre and their blood samples were collected from the caudal peduncle with means of vacutainer syringe and collected in a vacutainer tube which contains EDTA (anti-coagulant). The blood samples were poured into the centrifuge tube containing 1ml of saline solution (0.9 %). Then the samples were centrifuged at about 5,000 rpm for 30 minutes to separate the serum. The sera were stored at 4 ºC until use for the further analysis.

9.2.1. Quantification of Serum protein

The crude serum protein content of T. macrura was determined based on the methodology of AOAC (1995).

9.2.2. Gel permeation Chromatography

The crude serum protein of Moray eel (Thyrsoidea macrura) was fractionated through Sephadex G-25 column (5×90 cm) by the method of Cruz et al. (1987). Briefly, the lyophilized serum (10 mg) was dissolved in elution buffer (50 mM phosphate buffer, pH 7.4) and fractionation was carried out at 25°C at a flow rate of 0.5 ml/min and fractions were collected. The fraction was estimated for the total protein concentration by Lowry et al. (1951). The partially purified protein sample was subjected to further analysis.

9.2.3. SDS – Polyacrylamide gel electrophoresis (SDS–PAGE) and Amide band patterning of Serum protein

SDS page and FT-IR analysis were done by following the method described in Chapter 4.
9.2.4. NMR analysis of Serum protein

NMR is usually observed when the nuclei of certain atoms are placed in a static magnetic field, $^1$HNMR and $^{13}$C NMR spectra of serum protein were recorded using BRUKER 500 ultrashield™. $^1$H NMR of the peptide rich fraction has been magnified many fold. NMR spectroscopy was performed in serum sample of (5 mg) which is dissolved in D$_2$O (99.96%) and filtered through a 0.45 um syringe filter, freeze dried twice to remove the exchangeable protons and transferred to shigemi tubes. The experiments were carried out at room temperature in which the solvent peak does not interfere. The sample tubes was inserted in the magnet and allowed to reach thermal equilibrium for 10 minutes before performing the experiment. Similar process was repeated for $^{13}$C NMR.

9.2.5. Antimicrobial activity of Serum protein

9.2.5.1. Antibacterial activity

The spectrum of antibacterial activity was studied using serum protein against 6 different strains of human pathogenic gram negative and gram positive bacteria viz., *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Vibrio parahaemolyticus*, *Salmonella paratyphi* and *Klebsiella oxytoca*. In vitro antibacterial assay was carried out by disc diffusion technique (Bauer *et al.*, 1966). Whatmann No.1 filter paper discs with 6 mm diameter were impregnated with known amount of test samples (50 μl of serum protein) and standard antibiotic disc served as a positive control were placed on the Nutrient agar plates seeded with test bacterial strains. The antibacterial activity was expressed in terms of radius of zone of inhibition and was measured in mm using a scale and recorded.
9.2.5.2. Antifungal activity

Stock cultures of 6 different species viz., *Penicillium digitatum*, *Aspergillus flavus*, *Mucor* sp., *Aspergillus* sp., *A. niger* and *Penicillium* sp. were maintained in SDA broth for 48-72 hrs and used for antifungal activity. In vitro antifungal activity was determined by using the technique of Bauer *et al.* (1966) by employing 0.1 ml of 72 hrs old cultures maintained in SDA broth. Whatmann No.1 filter paper discs with 6 mm diameter were impregnated with known amount of test samples (50 μl of serum protein) and standard antibiotic disc served as a positive control were kept at the centre of the Potato dextrose agar plates seeded with test fungal strains. The inhibition zone was measured after 48 hrs and the Antifungal activity was measured in terms of radius of zone (including the disc within in mm).

9.2.6. Cytotoxic activity of Serum protein

9.2.6.1. Cell culture

Human breast adenocarcinoma cancer cell line MCF-7 was obtained from National Center for Cell science (NCCS), Pune. The cells were cultured in Eagles Minimum Essential Medium containing 100 unit/ml penicillin and 100 unit/ml streptomycin and supplemented with 10 % fetal calf serum at 37° C in a humidified atmosphere with 5 % CO₂, 95 % air and 100 % relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice in a week.

9.2.6.2. Cytotoxicity bioassay

Microculture tetrazolium assay is specifically meant to check the percentage of cytotoxicity for a drug or other bioactive substance. It is a colorimetric assay based on the activity of mitochondria succinate dehydrogenase enzyme in living cells to
reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-y1)-2, 5-diphenyl tetrazolium bromide (MTT) to an insoluble, coloured formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

MCF-7 cells were seeded in 96-well plates at the density of 2 x 10^5 cells per well. The cultured cells were incubated for 24 h for attachment, prior to addition of partially purified serum protein at different concentration 5, 10, 30, 50, 80 and 100 µg/ml, and then cells were incubated for 24 h. The control cells were maintained without addition of any drug. The cell survival rate was determined with addition of MTT (5 mg ml\(^{-1}\) in PBS) 20 µl per well and further incubated for 4 h at 37\(^{\circ}\)C. After pouring out the culture medium, 200 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple formazan product in living cell and measured by the absorbance at 492 nm (Mosmann, 1983). The percentage of cell viability was calculated against drug concentration.

\[
\text{Percentage of Cell viability} = \frac{[A]_{\text{Test}}}{[A]_{\text{control}}} \times 100
\]
9.3. RESULTS

9.3.1. Quantification of Serum protein

The total protein content of crude serum protein was 4.6 mg/ml whereas partially purified serum protein estimated was 3.8 mg/ml.

9.3.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE gel profile obtained for the standard (Lane 1) against the serum protein of Moray eel was depicted in Fig. 40. The serum protein was examined by using 12% gels. The Lane 1 represents the standard marker, Lane 2 represents the crude sample and comprises of three different molecular weight components which include 99, 89 and 78 kDa. The Lane 3 represents partially purified serum protein consisting of two different molecular weight components with 81 and 71 kDa.

![SDS PAGE analysis](image)

**Fig. 40.** SDS PAGE analysis of extracted serum protein

Lane 1- standard marker; Lane 2-crude Lane 3 partially purified protein
9.3.3. Amide band patterning of serum protein

Peak locations of FT-IR spectra were assigned for the serum protein of Moray eel in the wavelength of 400-4000 cm\(^{-1}\) and were represented in Fig. 41. The characterization of the chemical structure of any extracted compound can be confirmed by using Fourier transform infra-red (FT-IR) technique. The specific chemistries and orientation of the structure will be known from the IR spectrum. The infra-red spectra of the extracted compound were measured using the Perkin Elmer FT-IR model 2000 spectrophotometer. Background spectra, which were collected under identical conditions, were subtracted from the sample spectra automatically. Peak Fit (version 4.121) was used for data acquisition and handling. Direct comparisons of spectral changes were done on vector normalized second derivatives of average spectra of the discussed spectral classes, because most IR absorption bands are broad and composed of overlapping components. Second derivative spectra were vector normalized at 3500–3000 cm\(^{-1}\), at 1500-1800 cm\(^{-1}\), 1200 to1600 cm\(^{-1}\) and at 800–1200 cm\(^{-1}\). Fourier self-deconvolution and second derivative resolution enhancement were used to narrow the width of infrared bands and increase the separation of the overlapping components with software Peak Fit Version 4.12. The resolution enhancement resulting from self-deconvolution and the second derivative was such that the number and the position of the bands to be fitted were readily determined. Curve fitting was accomplished during a curve-fitting process with Peak Fit software (version 4.121) for the ester and amide carbonyl I, and II band region. The program affirms the curve-fitting process by adjusting the peak height and width to achieve the best Gaussian-shaped curves that fit the original spectrum. In the observed IR-frequency (vcm\(^{-1}\)) 3348.42 vcm\(^{-1}\) was assigned to the primary amine N-H, similarly in IR-frequencies 2887.44 to 2974.23 vcm\(^{-1}\) consigned...
alkane C-H stretching, stretching IR frequencies of 1570.06 -1658.78 corresponds to -C=O stretching. The frequencies of 1446.61, 1417.68 and 1384.89 cm\(^{-1}\) indicates CH\(_3\) bending absorption where IR-frequency 881.47 cm\(^{-1}\) refers to CH out of plan bending occurs.

Fig. 41. FT-IR analysis of serum protein extracted from *T. macrura*
9.3.4. NMR analysis of serum protein

The assigned NMR spectrum for the serum protein of Moray eel were shown in the Fig. 42 and 43. Whereas in the $^1$H-NMR number of proton was calculated based on the chemical shift which was presented here as $2[H]$ d with chemical shift at 1.191 ppm $2[H]$ d 1.341-1.326 ppm indicates methyl protons, $1[H]$s refers substituted methyl carbon containing protons. $2[H]$ d at 2.084, 2.827, 2.979 and 2.974 ppm indicated alkyl protons, chemical shift at 3.081-3.740 ppm refers to methoxy protons. 3.96-3.98 ppm chemical shift values signified the amide protons. In the case of the $^{13}$C NMR spectrum of serum protein from T. macrura showed the chemical shift at 20.00 and 22.81 ppm that evidents that methylenic carbon. The band at 68.39 ppm showed CH$_2$ aliphatic carbon. The band at 73.81 and 75.00 ppm represented the CH of aliphatic carbon. The band at 180.75 ppm indicated the carbonyl carbon for allylic C=O carbon.
Fig. 42. $^1$H NMR analysis of serum protein extracted from *T. macrura*

Fig. 43. $^{13}$C NMR analysis of serum protein extracted from *T. macrura*
9.3.5. Antimicrobial activity of Serum protein

In the present investigation, the serum proteins of eel exhibited a promising antibacterial activity. In this assay serum protein isolated and characterized from eel was screened for their ability to suppress/inhibit the growth of 6 human bacterial pathogens viz., *E. coli*, *S. aureus*, *Proteus mirabilis*, *V. parahemolyticus*, *K. oxytoca* and *S. paratyphi* (Fig. 44). The respective zone of inhibitions was depicted in Table 13. The serum protein exhibited a prominent susceptibility against *E. coli* with 12 mm of inhibition zone. The growth of *Proteus mirabilis* and *S. aureus* were inhibited to a greater extent by the serum protein and evidenced a zone of inhibition of 11 mm each, whereas moderate antibacterial activity was observed in *V. parahaemolyticus* with 9 mm. The serum protein revealed 7 mm zone of inhibition in *K. oxytoca* which was found to be comparatively less with other bacterial pathogens tested. Among the six pathogens tested the serum protein exhibited a least antibacterial activity of 5 mm against *S. paratyphi*.

Table 13. Antimicrobial activity of Serum protein extracted from *T. macrura*

<table>
<thead>
<tr>
<th>S. NO</th>
<th>Bacterial Pathogens</th>
<th>Zone of inhibition (mm)</th>
<th>Fungal strains</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>E. coli</em></td>
<td>12</td>
<td><em>P. digitatum</em></td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td><em>K. oxytoca</em></td>
<td>7</td>
<td><em>Penicillium sp.</em></td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td><em>S. paratyphi</em></td>
<td>5</td>
<td><em>A. niger</em></td>
<td>2</td>
</tr>
<tr>
<td>4.</td>
<td><em>S. aureus</em></td>
<td>11</td>
<td><em>Mucor sp.</em></td>
<td>3</td>
</tr>
<tr>
<td>5.</td>
<td><em>V. parahaemolyticus</em></td>
<td>9</td>
<td><em>Aspergillus sp.</em></td>
<td>2</td>
</tr>
<tr>
<td>6.</td>
<td><em>P. mirabilis</em></td>
<td>11</td>
<td><em>A. flavus</em></td>
<td>3</td>
</tr>
</tbody>
</table>
In antifungal assay isolated serum protein was tested against six fungal strains viz., *P. digitatum*, *A. niger*, *Penicillium* sp. *Mucor* sp. *A. flavus* and *Aspergillus* sp. (Fig. 45). The maximum susceptibility of 4 mm was observed in *Penicillium digitatum*. The isolated serum protein exhibited moderate antifungal activity on *Mucor* sp. and *Aspergillus flavus* and it represented a 3 mm of average zone of inhibition. The *Aspergillus* sp. and *A. niger* was found to be less susceptible to the serum protein with an inhibition zone of 2 mm. There was no inhibition zone observed in *Penicillium* sp.
Fig. 44. Antibacterial activity of serum protein extracted from *T. macrura*

Fig. 45. Antifungal activity of serum protein extracted from *T. macrura*
9.3.6. Cyotoxic activity of Serum protein

Effect of serum protein on cell proliferation was determined by MTT assay against MCF-7 cancer cell line. MCF-7 cells proliferation was significantly inhibited by serum protein extracted from *T. macrura*. The changes in the percentage (%) of cell inhibition in the untreated and serum protein treated at various concentrations of 5, 10, 30, 50, 80 and 100 µg/ml on MCF-7 cells were illustrated in Fig. 46.

In this study, the considerable cytotoxicity (79.31 %) was observed in serum protein treatment at a concentration of 100 µg/ml followed by moderate toxicity of 58.29 % (80 µg/ml) and 49.70 % (50 µg/ml). 30 and 10 µg/ml concentration of serum protein exhibited least toxicity 30.68% and 13.50 % respectively (Fig. 47). The inhibitory concentration 50 (IC$_{50}$) of serum protein was found to be as 55 µg/ml in MCF-7 cells.

![Fig. 46. Antiproliferative activity of serum protein extracted from *T. macrura*](image-url)
Control cells

5 µg/ml treated cells

10 µg/ml treated cells

30 µg/ml treated cells

50 µg/ml treated cells

80 µg/ml treated cells

100 µg/ml treated cells

**Fig. 47.** Cytotoxicity of serum protein extracted from *T. macrura* on MCF-7 cell line
9.4. DISCUSSION

Currently used anticancer drugs are mostly based on alkylating agents, anti metabolites as well as natural products and its derivatives. Between 1940 and 2006, 175 new anticancer drugs were approved (Newman and Cragg, 2007). More than 50% there of are biological (10% are usually large peptides and proteins), natural (14%) or naturally derived products (28% are semi synthetic modifications). A greater understanding of the mechanism of action of these molecules has potential for the development of human therapeutic agents.

9.4.1. Quantification of Serum protein

In the present study, the protein content was 4.8 mg/ml in crude and 3.6 mg/ml in purified serum protein of Moray eel, *Thyrsoidea macrura*. The results of the present study can be correlated with Lee *et al.* (2011) recorded 4 mg/ml of serum protein in sand eel, *Hypophtychus dysbowskii*. The obtained results are in same line with Kumaravel (2010) who revealed that the protein content in haemolymph of crab *Eurypanopeus orientalis* was found to be 3.6 mg/ml. The protein content was found to be 4.4 mg/ml in the crab, *Dromia dehaani* by Sylverster (2013). Yilmaz *et al.* (2007) reported that the protein content of *Leuciscus cephalus* was found to be 2.9 mg/ml. Dhanalakshmi *et al.* (2012) reported that the protein content in marine sponge of *Callyspongia* sp. was around 2.9 mg/ml. Boobathy *et al.* (2009) obtained the crude protein content of 1.62 mg/ml in marine sponge *Callyspongia diffusa*. 
9.4.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

In the present study, the molecular weight of the partially purified serum protein of *Thyrsoidea macrura* was estimated with two different molecular weight components with 81 and 71 kDa. This molecular weight was lower than the peptide of conotoxin isolated from *C. betulinus* (Giji *et al.*, 2014). Saravanan *et al.* (2009) have reported two different peptides with low molecular weight of about 14 kDa. The present study showed the molecular weight of the partially purified serum of *Thyrsoidea macrura* was higher than the molecular weight of hydrophobic protein of carp with molecular weight of 27 and 31 kDa (Ebran *et al.*, 1999). Relf *et al.* (1999) isolated an antibacterial protein with molecular weight of 11.5 kDa from haemocytes of shore crab, *Carcinus maenas*.

9.4.3. Amide band patterning of serum protein

The Fourier-transform infrared (FT-IR) spectra (4000 – 400 cm\(^{-1}\)) were obtained by employing KBr pellets on a Nicolet 210 FT-IR spectrophotometer. The KBr pellets were prepared by grinding 15 mg of each sample with 300 mg of solid KBr (previously dried at 120°C for at least 2 hours). The mixture was transferred to a punch and dye system and a pressure of 15,000 pounds were applied for 10 minutes using a hydraulic press, where a transparent pellet was obtained. OMNIC software was employed for data analysis and the number of scans and resolution were 120 and 8 cm\(^{-1}\), respectively. FT-IR spectroscopy identifies the molecular structures that are present in a substance based on their respective absorption bands in the infrared spectrum. As a consequence characteristics of bioactive compound are usually performed by using FT-IR. It can be used for conformational analysis of peptides in a wide range of compounds. The amide primary amine of serum protein was found at 3348 cm\(^{-1}\). The absorption characteristics of primary amine are commonly associated...
with N-H stretching vibrations that occur in the wave number range of 3400-3440/ cm\(^{-1}\) (Sai and Babu, 2001). The stretching of IR frequencies are 1570.06 -1658.78 that corresponds to the -C=O stretching in the present study. This is the sensitive area of changes of protein structure analysis and often used for predicting secondary structure of protein. This result was correlated with the report in haemolymph of crab who stated that primary amine observed at the same range of 3420 cm\(^{-1}\) by Sylvester (2012). The present study confirms the presence of amine group by FT-IR analysis of serum protein.

**9.4.4. NMR analysis of Serum protein**

Over the next 50 years NMR will be developed into the premier organic spectroscopy available for chemists to determine the detailed chemical structure of the chemicals they were synthesizing. Another well-known product of NMR technology has been the Magnetic Resonance Imager (MRI), which is utilized extensively in the medical radiology field to obtain image slices of soft tissues in the human body. In recent years, NMR has moved out of the research laboratory and into the on-line process analyzer market. This has been made possible by the production of stable permanent magnet technologies that allow high-resolution \(^1\)H NMR spectra to be obtained in a process environment. The NMR phenomenon is based on the fact that nuclei of atoms have magnetic properties that can be utilized to yield chemical information. Quantum mechanically subatomic particles (protons, neutrons and electrons) have spin. In some atoms (eg \(^{12}\)C, \(^{16}\)O, \(^{32}\)S) these spins are paired and cancel each other out so that the nucleus of the atom has no overall spin. However, in many atoms (\(^1\)H, \(^{13}\)C, \(^{31}\)P, \(^{15}\)N, \(^{19}\)F etc..) the nucleus does possess an overall spin.
From a purely intellectual viewpoint, one of the fascinating things about nuclear magnetic resonance (NMR) is the complexity of the subject. However, this complexity can be the source of much frustration for those wishing to understand and use NMR. As with other physical techniques used in studies of biological systems, NMR may be used in an empirical mode; for example, simply noting variations in an NMR parameter with alteration of an experimental variable. However, a better understanding of the NMR phenomenon is often rewarded with additional elucidation of the system under study. Although there may be thresholds of knowledge of NMR necessary to read the literature critically or to conduct NMR studies, there is a continuum of knowledge to be gained about NMR that has the practical dividend of enabling an ever greater variety of NMR experiments to be utilized correctly.

In the present study, presence of protein can be confirmed through the chemical shift at 2[H] d at 2.084, 2.827, 2.979 and 2.974 that indicates alkyl protons, chemical shift values for 3.081-3.740 refers to methoxy protons whereas 3.96-3.98 chemical shift values indicates amide protons. $^{13}$C NMR shows, δ 20.00 and 22.61 are evident that methylenic carbon. The band at 73.81 and 75.00 ppm represented the CH of aliphatic carbon. The band at 180.75 ppm indicated the carbonyl carbon for allylic C=O carbon. Similarly the presence of peptide was confirmed by $^1$H NMR in the haemolymph of Ocypoda macrocera (Ravichandran et al., 2010). The crab Thalamita crenata, peptide is confirmed by the presence of doublets in the region of its NMR spectrum (Rameshkumar et al., 2009a). Kumaravel (2010) reported the purified haemolymph sample of Euryphanopeus orientalis crab shown the wavelength range of 7.229 ppm by $^1$H NMR and $^{13}$C NMR in range of 77 ppm. The results of NMR confirms the presence of amine group containing carbon atom and also affirms the presence of amide protons.
9.4.5. Antimicrobial activity of Serum protein

Even though the advanced improvements and new formulations in the modern chemotherapeutic techniques have been vastly studied and applied, the infectious pathogens still remains a major public health issue worldwide. In addition to that the multi drug resistance or in other words resistance to many of the commonly used antibiotics among the pathogenic microbes hinders the potential of active compounds to a greater extent. To combat this, nowadays research is focused on identification of effective compounds especially from marine origin. The results of the presents study reveals that the serum proteins evidenced a better antibacterial activity against *E. coli, P. mirabilis and S. aureus*. These results are in agreement with the report of Wang *et al.* (2011) who had proven that the serum protein (SR-LAAO) of rabbit fish inhibits the growth of *S. aureus* and *E. coli*. The growth of *P. mirabilis* (11 mm), *K. oxytoca* (7 mm) were suppressed and this can be correlated with the inferences of Rameshkumar *et al.* (2009a) who reported that the antimicrobial peptide was isolated for the first time from the haemolymph of the crab *Thalamita crenata* which expressed a moderate antibacterial activity. *Proteus mirabilis* (8 mm) and lowest zone of inhibition was observed against *Klebsiella oxytoca* and *Lactobacillus vulgaris* (7 mm). Fish display a variety of AMPs, most of them are positively charged short amino-acid chains playing a key role in host defense mechanisms. Hajirasouli and Pazooki (2014) recorded the antimicrobial potential of hemolymph and hepatopancreas of *Portunus segnis* crabs to be maximum of 8 mm and minimum of 3.6 mm against *Entrobacter* sp. and *Serratia marcesences* respectively. Grinde (1989) reported on the antibacterial effect of two lysozyme variants purified from rainbow trout *Salmo gairdneri* showed antibacterial effect on human pathogens.
Relf et al. (1999) isolated an antibacterial protein, cysteine rich 11.5 kDa from granular haemocytes of the shore crab, *Carcinus maenas* and the isolated protein acts against both gram negative and gram positive bacteria such as *S. aureus, S. epidermidis, Planococcus citreus, Planococcus kocurii*.

In case of antifungal assay the serum protein revealed a moderate antifungal activity against the fungal starins tested. The moderate zone of inhibition was observed against *A. niger, Mucor* sp., *A. flavus* and the results are in agreement with Venkatachalam Uthayakumar et al. (2012). These authors recorded the mucus extracts of fresh water spiny eel *Mastacembelus armatus* displayed a nominal inhibition of *A. niger, Mucor* sp., *A. flavus*. Dhanalakshmi et al. (2012) had investigated the invitro antimicrobial activity and bioactivities of protein isolated from marine sponge and evidenced that they suppressed the growth of *Aspergillus niger* and *Candida albicans*. The mucus extracts from *Catla* and Silver carp displayed a medium antifungal activity against *A. niger* and *A. flavus* (Balasubramanian et al., 2012). The serum protein didn’t showed any inhibitory effect on *Penicillium* sp. and this lies parallel to the statement of Rameshkumar et al.(2009a) who reported that the peptide isolated from the Crab, *Thalamita crenata* has no symptoms of antifungal activity.

The isolated serum protein exhibits a promising antibacterial property and shows a moderate antifungal activity; comparatively they could be better utilized against bacterial pathogens. These peptide may play a role in protection against intracellular or extracellular pathogens (Fernandes and Smith, 2002). Fish display a variety of AMPs, most of them are positively charged short amino-acid chains playing a key role in host defense mechanisms The serum protein Pentraxin: C reactive protein (CRP) and serum amyloid protein (SAP) appear to be expressed continuously
in fishes and shows a relatively high concentration in serum of amount (50-300 µg/ml) and are expected to play a vital role in defense mechanism and more research is needed (Lund and Olafsen, 1999). Several attempts have been made to explore the new antimicrobial drugs from marine natural sources. The present study indicates that the serum protein of Eel would be a good source of antimicrobial agents and would replace the existing ones.

9.4.6. Cyotoxic activity of Serum protein

In recent years, peptides have been widely utilized as a material for food additives, cosmetics, biomedical materials and pharmaceuticals due to their excellent biocompatibilities and biodegradabilities, and weak antigenicities. Cytotoxicity can be assessed by different methods such as microscopical evaluation of cell morphology and Methyl tetrazolium assay (MTT). The percentage of cell viability count to determine cytotoxicity activity against MCF-7 cell line is represented in Fig. 46. The varying concentrations of serum proteins viz., 5, 10, 30, 50, 80 and 100 µg/ml were tested against MCF-7 cell line to explore its cytotoxic effect.

The cell viability was found to be 22.69% in100µg/ml and it evidenced the dose dependent decrease in the viability of cancer cells at the incubation time of 48 hrs. The isolated serum protein showed maximum cytotoxicity effect at 100 µg/ml and inhibitory concentration was found at 79.3 % whereas minimum effect was observed in 30 and 10 µg/ ml concentrations (30.68% and 13.50%) against MCF-7 cancer cell line.

The effects of the morphology of the cancer cells are given in the photoplate. Using MTT assay such cytotoxicity of serum protein was characterized by the close different or reflected by the correspondingly low IC₅₀ values was observed with
50.30 % at 55 µg/ml. The results of the present study is in same line with the reports of Lin (2014) who isolated and identified a novel linear polypeptide named mere15 from marine mollusks *Meretrix meretrix* which suppress the growth of human lung adenocarcinoma. An anti tumour protein from coelomic fluid of *Meretrix meretrix* exhibited cytotoxicity to cancer MCF-7 (Fouda, 2005). Many authors reported that antimicrobial peptide showed both anti tumour and anticancer activities from marine fishes (Risso *et al.*,1998; Fouda, 2005; Hoskin and Ramamoorthy,2008).

The antimicrobial peptide *Syngnathusin* from *Syngnathus acus* showed antitumor activity by inducing apoptosis at A549 and CCRF-CEM cells (Wang *et al.*, 2012). Pedrino *et al.* (2004)isolated potent anticancer compounds from fish tissue and it is a strong argument to consider that byproducts could constitute a source of anticancer or chemopreventive molecules. Fish hydrolysates of Blue whiting, salmon and cod constitutes of 96% protein which showed antiproliferative activity on MCF-7 cancer cells, it could be related to presence of specific peptides exerting direct cytotoxicity. Picot *et al.* (2006) also evaluated the efficacy of haemoglobin derived peptide Valorphin against antiproliferative activity. The hydrophobic peptide (440 kDa) isolated from anchovy was able to induce apoptosis in human U937 lymphoma cells through the increase of caspases activity (Lee *et al.*, 2004). Antiproliferative peptides purified from tuna dark muscle by enzymatic hydrolysis with papain and protease showed inhibitory effects against cancer cell line MCF-7 as reported by Hsu *et al.* (2010).

The serum protein isolated from blood of Moray eel showed best cytotoxicity against MCF-7 cancer cell line of 79.31% at 100 µg/ml. Laursen *et al.* (1990) reported that the growth of the estrogen responsive cancer cell line, MCF-7, is inhibited by
high serum concentrations. This growth inhibition can be abolished by estradiol (E2).
This result was in agreement in the present study; cell line growth was inhibited in high concentration (100 µg/ml) of serum protein. The present study demonstrates that the serum protein could significantly suppress the proliferation of MCF-7 cell line. Ranathunga *et al.* (2006) purified antioxidative peptide, tryptic hydrolysate with molecular mass of 935 kDa from conger eel (*Conger myriaster*) and it scavenged the hydroxyl radicals and carbon-centered radicals at IC50 values of 74.1 µM and 78.5 µM, respectively. Lee *et al.* (2011) characterized an antioxidant peptide and reported that the free radical scavenging activity of papain hydrolysate was 77.4 % at 1.0 mg/ml. There is no doubt that the serum protein can be used as the potential drug for cytotoxicity to MCF-7 and can also extend to various pharmaceutical applications.