Int.J.Curr.Microbiol.App.Sci (2017) *6*(4): 159-167

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 4 (2017) pp. 159-167**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2017.604.018

Identification of Protein from Muscle Tissue of Marine Finfish

B. Deivasigamani*, Vasuki Subramanian and A. Sundaresan

Faculty of Marine Sciences, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai – 608 502, Tamil Nadu, India
**Corresponding author*

Keywords

Protein, Finfish, Nectar, *Mystus gulio*, FTIR and GCMS

Article Info

Accepted:
15 March 2017
Available Online:
10 April 2017

A B S T R A C T

The isolation of natural products from marine fish includes several essential steps. The process begins with the isolation of tissues from the fish. Often, in the past, the isolation of compound has been a random process. However there is now a growing recognition that the source of fish samples can be important for increasing the success rate of bioactive discovery. Due to the various and often chemically mediated interactions that occur between tissues and their host and between members of the fish community, isolation of compound from marine finfish can significantly increase the chances of obtaining bioactive producing strains. The present study was to evaluate the antimicrobial activity of *Mugil cephalus* Muscle Tissue and proteins were tested by using disc diffusion techniques against seven pathogenic bacteria.

Introduction

The ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity. The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural and chemical features not found in terrestrial natural products. The richness of diversity offers a great opportunity for the discovery of new bioactive compounds. The number of natural products isolated from marine organisms increases rapidly and now exceeds with hundreds of new compounds being discovered every year. Now a day the development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for further attempts to search for new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents. Action must be taken to reduce this problem such as, controlling the use of antibiotics, carrying out research to investigate drugs from natural sources and also drugs that can either inhibit the growth of pathogen or kill them and have no or least toxicity to the host cell are considered conditions for developing new antimicrobial drugs. The main aim of this work is to identify the marine finfish compounds. The number of natural products, discovered from various living organisms including plants, animals and microbes, to

date exceeds 1 million, with the majority (40–60%) derived from terrestrial plants (Capon, 2001). Of these natural products, 20–25% possesses various bioactive properties including antibacterial, antifungal, antiprotozoal, antinematode, anticancer, antiviral and anti-inflammatory activities (Pelaez and Genilloud, 2001).

Plants and plant extracts have been used for the treatment of human diseases for millennia, and their use has been recorded in the most ancient archaeological sources (Berdy, 2005). In contrast, the exploration of microorganisms as producers of therapeutical agents only began in the 20th century (Monaghan and Tkacz, 1990). However, despite this relatively short history, nearly 10% of all currently known biologically active natural products are of microbial origin. These include the majority of antibiotics, clearly demonstrating the potential of microorganisms as an emerging source for the production of biologically active products. Indeed, by the 20th century microbially derived bioactives had become the foundation of modern pharmaceuticals. For example, the production of antimicrobials is observed in 30–80% of actinomycete and fungal strains screened in various studies (Fenical and Jensen, 2006). Moreover, mathematical models predict that the number of undiscovered antibiotics from actinomycetes could be in the order of 10^7 (Basilio *et al.,* 2003).

An emerging source of new bioactives may result from the many recent studies of microbial diversity in the marine environment, particularly those microbes associated with marine plants and animals. Several studies have demonstrated that "living surfaces" represent an environment rich in epibiotic microorganisms that produce bioactives (Longford *et al.,* 2007). Nevertheless, the vast biotechnological potential of marine epibiotic microorganisms remains mostly unexplored (Santiago *et al.,* 2007, Rheinheimer, 1992, Perez-Matos *et al.,* 2007).

Mystus gulio is commonly used as a food fish and has occasionally been caught and exported as an ornamental fish (Ng, 2010). It is an important target species for small scale fishermen and artisanal fisheries who use a variety of traditional fishing gears (Begum *et al.,* 2008; Ravindra and Thilina, 2010; Ng, 2010). This small indigenous fish contains a high nutritional value in terms of protein, micronutrients, vitamins and minerals which are not usually found in other foods, making it a very favorable candidate for aquaculture in Southeast Asia (Ross *et al.,* 2003). Fish is an excellent and relatively a cheaper protein source of high biological value (Watve *et al.,* 2001, Ulfat Jan *et al.,* 2012).

A number of naturally occurring antimicrobial proteins have been characterized from fish skin, muscle and gills, such as piscidins, but these and other fish tissues may contain numerous other compounds with bioactive properties. Such compounds could be extracted by the subsection of the fish industry that processes marine secondary products and further developed to commercial products. Thus, the identification of novel bioactive compounds from fish could be utilized by the pharmaceutical and biotech industry to develop new products. The aim of this study is to characterize the bioactive compounds present in *Mugil cephalus* muscle tissue by using FTIR, GC MS and SDS PAGE analysis.

Achieved objectives

Samples were collected from South East coast of Tamil Nadu and various solvent extracts were prepared from the sample to study its antimicrobial activity under various concentrations.

Characterize the extracts showing antimicrobial activity using analytical techniques.
Novel compounds was characterized by FTIR and GCMS
Abstract and paper was published by reviewed journal

The objective of the present study was to evaluate the antimicrobial activity of *Mugil cephalus* muscle Tissue. Fishes are in relation to aquatic habitat, which contains very high concentrations bacteria and viruses. The immune system is composed of numerous organs and cells that act together in a dynamic network in the defense against infection, disease and foreign substances. Fish proteins were tested by using disc diffusion techniques against seven pathogenic bacteria such as *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella pneumonia*. The activity was measured in terms of zone of inhibition in mm. The protein from *Mugil cephalus* showed broad spectrum of antibacterial activity.

Materials and Methods

Fish collection and acclimatization

Live fish, *Mugil cephalus*, was purchased from the nearby fish landing center and local fish market and maintained in circular plastic fish tanks (1000 L capacity) at Fisheries Laboratory, CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, India. The fish acclimatized to laboratory conditions in a fish tanks and they were maintained for one week. During this period the fish were fed with commercial feed once a day at *ad libitum*. Half of the water of the tank was changed on alternate days. Dissolved oxygen was maintained at a preferable level in the tank with the help of low-pressure aerators and pumps. The health of fishes was observed daily, and dead fish or fish with lesions (if any) was immediately removed.

Preparation of *Mugil cephalus* muscle tissue

The fishes were washed, beheaded, sliced and covered with ice to ensure freshness of the fish tissues. The fish muscle tissue was then sliced into smaller pieces and placed in sterile universal bottles and kept at -20°C prior to freeze-drying. Freeze dried *Mugil cephalus* muscle tissue was homogenized to powder form. Extraction of protein from *Mugil cephalus* muscle tissue was carriedout on 1.0 mg of powdered fish muscle using 1 mL of 40mM Tris (pH 8.8) extraction buffer. The sample mixture was then vortexed for 2 minutes and centrifuged at 12, 000 g for 30 min at room temperature and the supernatant was recovered.

Protein estimation

The protein concentration of the samples was determined by the method of (Lowry *et al.*, 1951) with bovine serum albumin as standard. To 5ml of Lowry reagent, add 1ml of suitably diluted sample and the mixture was kept at room temperature for 10min. To this add 0.5ml of Folin's reagents and kept at dark condition for 30mins. The absorbance was taken at 640nm.

Fourier transform - Infrared Spectroscopy (FT-IR) analysis

The *Mugil cephalus* muscle tissue samples were taken in the foam of fine powder (Saifuddin *et al.*, 2009) and were filtered with sieves of 0.071 and 0.500 mm mesh size. The FT-IR spectra were recorded in mid IR region 4000-400 cm-1 at the resolution of 4 cm-1 using a sophisticated computer controlled FT-IR Perkin Elmer spectrometer with He-Ne laser as reference. Air back ground spectrum was recorded before each sample.

GC-MS analysis

The GC-MS analysis of the *Mugil cephalus*

muscle tissue was performed using a Clarus 680 Perkin Elmer gas chromatography equipped with an Elite-5 capillary column (5% diphenyl, 95% dimethyl polysiloxane) (30.0m × 0.25mmID × 250 μm) and mass detector turbo mass of the company which was operated in EI mode. Helium was the carries gas used at a flow rate of 1 mL/min. The injector was operated at 200◦C and the oven temperature was programmed as follows: 60◦C for 2min and 10◦C/min until 300◦C. Interpretation of GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

SDS-PAGE

The protein profile of *Mugil cephalus* muscle tissue was analyzed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by as described by Laemmli(1970).Protein samples (6 µg total protein) were diluted 1:1 with sample buffer [4% (w/v) SDS, 50 mM Tris–HCl, 2% mercaptoethanol (v/v), 12% (v/v) glycerol and 0.5% (w/v) bromophenol blue adjusted with HCl to pH 6.8] and loaded onto a separating gel of 15% acrylamide with a 10% acrylamide spacer gel and 4% stacking gel. The gel was run in a Bio-Rad electrophoresis apparatus for 3.5 to 4 h at 90 V. SDS-PAGE standard markers (Low range, Bio-Rad laboratories Inc., CA, USA) were included to estimate the molecular mass of proteins. Proteins were visualized using silver staining (Blum *et al.*, 1987).

Antimicrobial assays

Five Gram-negative (*Escherischia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae)* and one Gram-positive organism (*Staphylococcus aureus*) were used for the study. *In vitro* anti-bacterial activities of the test samples were carried out by disc diffusion method (Bauer *et al.*, 1966). One antibiotic, Gentamycin was used against pathogenic bacteria as control. Bacteria were incubated in Nutrient broth for 24 h at 37 °C in a shaker and were adjusted to yield approximately 108 CFU/ml.

The inoculum was spread on Muller Hinton agar and air-dried at room temperature. A 6-mm sterile paper disc was impregnated with different concentrations of (25, 50, 75 and 100µl) *Mugil cephalus* muscle tissue extract and the disc were placed on the agar. The plates were left to dry and incubated at 37 °C for 24 h under aerobic condition. The results were recorded by measuring the zone of inhibition surrounding the disc. Clear inhibition zones around the discs indicated the antibacterial activity. The results obtained were expressed as the means ± SE of six values. Statistical analysis of the data was performed by DMRT (Duncan Multiple Range Test).

Results and Discussion

Protein content in *Mugil cephalus* muscle tissue

The protein content of the muscle tissue extract of *Mugil cephalus* was presented in table 1. The protein contents in the muscle extract of *Mugil cephalus* 27.20 (µg mL-1).

IR-spectroscopic analysis of *Mugil cephalus* muscle tissue

The IR spectral data of *Mugil cephalus* muscle extract showed a broad peak in the region of 3313.71 cm−1 where hydroxyl (-OH) and amide (-NH) group stretch was observed. The ester and ketone (C═O) group

stretch was observed in the region of 1604.77 cm^{-1}. The (C═N) stretching frequency was observed in the region of 1,515.59 cm^{-1} (Figure 1).

GC MS analysis of *Mugil cephalus* muscle tissue

Mugil cephalus muscle tissue has been analyzed by GC-MS technique. The results are given in table 3. The *Mugil cephalus* muscle tissue was shown to contain a mixture of components. Eleven components were identified. The analysis of *Mugil cephalus* muscle tissue showed 1,1-Dichloropentane, Ether, 3-Butenyl Propyl, Cyclohexanol, Bisnorallocholanic Acid, 4-Hexadecen-6-yne, Limonen-6-OL, Pivalate, Caryophyllene Oxide, Methoprene, 5,9-Undecadien-1-yne, Cyclotrisiloxane and Carvone Oxide CIS shown in table 3 and figure 2.

Table.1 The protein content of *Mugil cephalus* muscle tissue

Fish species	Protein content (µg mL)
Mugil cephalus	27.20 ± 12.42

Value is the mean and standard deviation of three replicates. Values followed by a different superscript letter on the same column are significantly different ($p < 0.05$)

Table.2 FTIR peak values and functional groups of *Mugil cephalus* muscle tissue

S. No	Peak area	Bond	Functional group
1.	3313.71	O-H stretch	Free hydroxyl alcohols phenols
2.	2920.23	C-H stretch	Alkynes
3.	2850.79	N–H stretch	1˚, 2˚ amines, amides
4.	1730.15	H–C=O: C–H stretch	Aldehydes
5.	1604.77	C=O– stretch	Ester
6.	1367.53	N-H Bend	Amines
7.	1232.51	C–N stretch	aromatic amines
8.	1010.70	N–O symmetric stretch	nitro compounds
9.	767.67	C–O stretch	Alcohols, carboxylic acids, esters, ethers
10.	524.64	C–N stretch	aliphatic amines

Figure.1 FTIR spectrum of *Mugil cephalus* muscle tissue

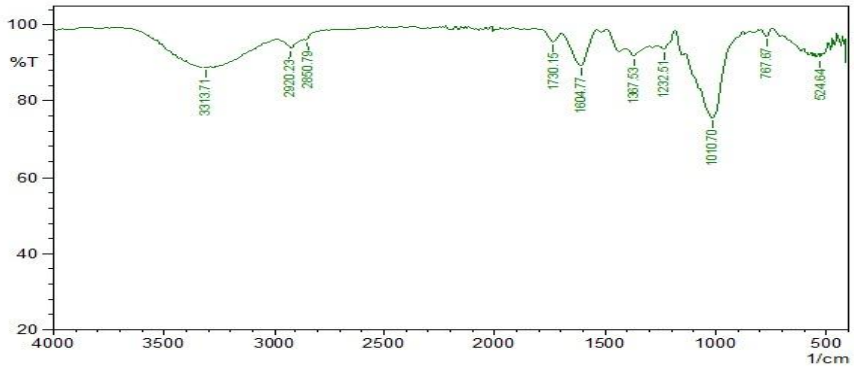


Table.3 GC MS analysis of *Mugil cephalus* muscle tissue exact

S. No	RT	Name of the compound	Molecular formula	Molecular Weight
1	2.523	1,1-DICHLOROPENTANE	140	$C_5H_{10}C_{l2}$
2	2.633	ETHER, 3-BUTENYL PROPYL	114	$C_7H_{14}O$
3	16.934	CYCLOHEXANOL	184	$C_{12}H_{24}O$
4	18.665	BISNORALLOCHOLANIC ACID	332	$C_{22}H_{36}O_2$
5	19.660	4-HEXADECEN-6-YNE, (E)-	220	$C_{16}H_{28}$
6	21.056	LIMONEN-6-OL, PIVALATE	236	$C_{15}H_{24}O_2$
7	21.906	CARYOPHYLLENE OXIDE	220	$C_{15}H_{24}O$
8	24.212	METHOPRENE	310	$C_{19}H_{34}O_3$
9	24.307	5,9-UNDECADIEN-1-YNE	176	$C_{13}H_20$
10	26.818	CYCLOTRISILOXANE	222	$C_6H_{18}O_3Si3$
11	27.253	CARVONE OXIDE, CIS-	166	$C_{10}H_{14}O_2$

Figure.2 GC MS analysis of *Mugil cephalus* muscle tissue

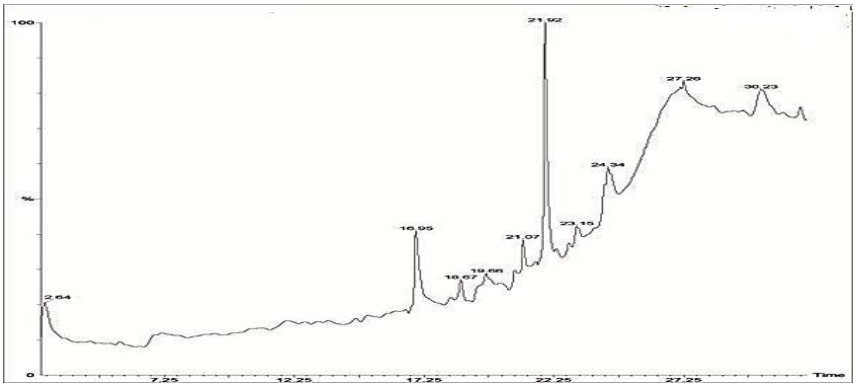
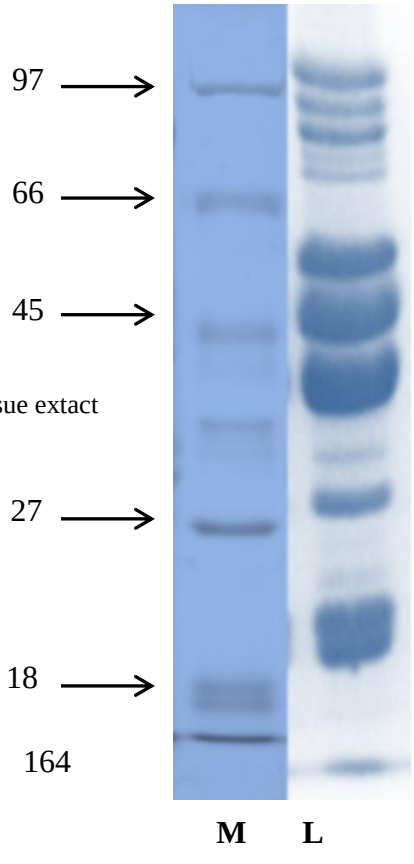
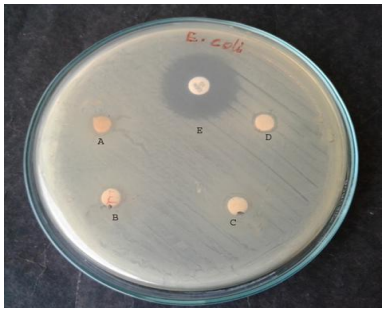


Figure.3 SDS-PAGE showing protein profile of *Mugil cephalus* muscle tissue exact

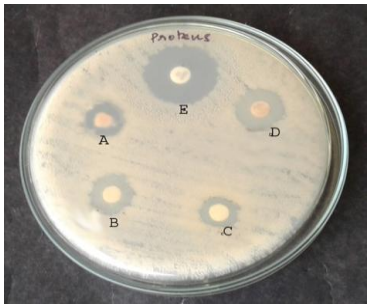


M - Low range molecular mass (kDa) marker.
L - Lane contains 50 µg of *Mugil cephalus* muscle tissue extact

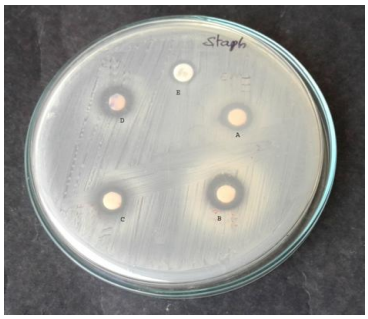
Figure.4 The antibacterial effect of *Mugil cephalus* muscle tissue against some bacterial pathogens



Escherichia coli

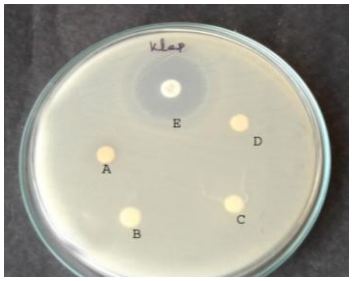


Proteus mirabilis



Staphylococcus aureus

Pseudomonas aeruginosa



Klebsiella pneumonia

A: 25 µl of mucus extract
B: 50 µl of mucus extract
C: 75 µl of mucus extract
D: 100 µl of mucus extract
E: 30 µl of Gentamycin

Protein profiles of *Mugil cephalus* muscle tissue

The protein profiles of *Mugil cephalus* was showed in figure 3. The SDS-PAGE profile showed the protein ranging from 100 kDa to less than 10 kDa.

Antibacterial activity

The present study was aimed to evaluate the in *vitro* antimicrobial activity of tissue extract of *Mugil cephalus* against five cultures namely *E. coli*, *P. mirabilis*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae* (Table 2). The tissues collected from fish showed a strong inhibition in the growth of tested bacteria. Clear inhibition zones around the discs indicated the presence of antimicrobial activity, however, the extracts differ in their activities against the microorganisms tested. A maximum zone of inhibition was observed against *P. mirabilis* (29 mm in diameter), followed by *S. aureus* with inhibition zone of 16 mm respectively. *P. aeruginosa* showed minimum (4.14 mm) inhibitory activity than other organisms.

In the present study, *Mugil cephalus* muscle tissue quantification results revealed that

muscle of fish contained a high amount of proteins. Further SDS PAGE analysis indicated that muscle protein contains protein ranging from 100 kDa to less than 10 kDa. In the silver staining method, many researchers isolated proteins from different tissue from various fishes: *Atlantic hagfish* (Park *et al.*, 1997), *Winter flounder* (Cole, Weis and Diamond, 1997), *Atlantic halibut* (Birkemo *et al.*, 2003).Fish is rich in protein with amino acid composition very well suited to human dietary requirements comparing favorably with egg, milk and meat in the nutritional value of its protein (Olomu, 1995).

The FTIR analysis showed distinct spectral profile confirming the presence of primary amine group, aromatic compound, halide group, and aliphatic alkyl group. In addition GC MS analysis showed sharp peak values between 2.03 and 56.39in the muscle of the fish. This result showed the presence of bioactive compounds present in the *Mugil cephalus* muscle tissue.

Usage of natural chemicals is an ancient practice in human civilization. Exploration of natural Compounds from different sources is a continuous task to improve and enrich their own lives (Agosta, 1996). Extracts and preparation made from the animal origin has been a great healing tool in folk and modern medicine (Kuppulakshmi *et al.*, 2005). The development of resistance by a pathogen of many of the commonly used antibiotics provide an impetus for further attempts to search for new antimicrobial agents which combat infections and overcome the problems of resistance with no side effects. In the present study, the inhibitory effect of the *Mugil cephalus* muscle tissue may be due to the poreforming properties against several bacterial strains and this suggested that fish secrete antibacterial proteins which act as an antimicrobial properties. The antibacterial activity may be due to the protein or glycol-proteins present in the fish that are able to kill bacteria by forming large pores in the target membrane (Ebran *et al.*, 1999; Park *et al.*, 1997; Manivannan *et al.*, 2011). Further studies on the characterization of the antimicrobial substances in these *Mugil cephalus* muscle tissue will further our understanding of the composition and function of the antimicrobial protein.

In conclusion marine organisms are currently accepted as the best renewable source for bioactives, and the exploration of yet underexplored sources, such as the marine living-surface habitat, has a great potential to deliver novel bioactive producing marine finfish tissues will useful for further drug development. Moreover, a systematic approach that takes into consideration unique ecological relationships in the marine environment, such as those discussed in this project, can greatly assist in maximizing the output of obtaining novel bioactive producing fish organisms and, thus, may prevent the frequent re-discovery of known compounds and the waste of resources that would be necessary for large scale high-throughput screens.

Acknowledgement

The authors dedicate their sincere gratitude to Dean and Director of CAS in Marine Biology, for their constant encouragement and support. The authors also thank GUCC/XII PLAN/2016, Annamalai University for the financial assistance.

References

Agosta W. (1996). Bombardier beetles and fevertrees: a close up look at chemical warfare and signals in animals and Plants, New York: Addison-wiselyPublishing Company, p.224.

Basilio, A; Gonzalez, I; Vicente, MF; Gorrochategui, J; Cabello, A; Gonzalez, A; Genilloud, O. (2003) Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. *J Appl*

Microbiol, *95*, 814–823.

Begum, M., Alam, M. J., Islam, M. A., Pal, H. K. (2008): On the food and feeding habit of an estuarine catfish (*Mystus gulio*) in the south-west coast of Bangladesh. Journal of Zoology, Rajshahi University, 27, 91-94.

Berdy, J. (2005) Bioactive microbial metabolites. A personal view. *J Antibiot (Tokyo)*, *58*, 1–26.

Birkemo GA, Lüders T, Andersen Ø, Nes IF, Nissen-Meyer J. Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). Biochim Biophys Acta 2003; 1646: 207-15.

Blum, H., Beier, H., Gross, H.J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99.

C. Kupulakshmi, M. Prakash G. Gunasekaran, G. Manimegalal, S. Sarojini (2008). Antibacterial properties of fish mucus from *Channa punctatus* and *Cirrhinus mrigala*. European Review for medical and Pharmacological Sciences. 12: 149-153.

Capon, RJ. (2001) Marine bioprospecting–Trawling for treasure and pleasure. *European J Org Chem*, 633–645.

Cole AM, Weis P, Diamond G. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J Biol Chem 1997; 272: 12008-13.

Ebran N, Julien S, Orange N, Saglio P, Lemaitre C, Molle G. Poreforming properties and antibacterial activity of proteins extracted from epidermal mucus of fish. Comp Biochem Physiol A Mol Integr Physiol 1999; 122: 181-9.

Fenical, W; Jensen, PR. (2006) Developing a new resource for drug discovery: Marine actinomycete bacteria. *Nat Chem Biol*, *2*, 666–673.

Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685 (1970).

Longford, SR; Tujula, NA; Crocetti, GR; Holmes, AJ; Holmstrom, C; Kjelleberg, S; Steinberg, PD; Taylor, MW. (2007) Comparisons of diversity of bacterial communities associated with three sessile marine eukaryotes. *Aquat Microb Ecol*, *48*, 217–229.

Manivannan K, Karthikai Devi G, Anantharaman P, Balasubramanian T. Antimicrobial potential of selected brown seaweeds from Vedalai coastal waters, Gulf of Mannar. Asian Pac J Trop Biomed 2011; 1: 114-20.

Monaghan, RL; Tkacz, JS. (1990) Bioactive microbial products: Focus upon mechanism of action. *Annu Rev Microbiol.*, *44*, 271–301.

Ng, H. H. (2010): Mystus gulio. The IUCN Red List of Threatened Species. Version 2014.2.

Olomu, J.M., 1995. Monogastric Animal Nutrition Jachem Publications, Benin City, pp: 165-200

Park CB, Lee JH, Park IY, Kim MS, Kim SC. A novel antimicrobial peptide from the loach, Misgurnus anguillicaudatus. FEBS Lett., 1997; 411: 173-8.

Pelaez, F; Genilloud, O. (2001) Discovering new drugs from microbial natural products. In: *Microorganisms for Health Care, Food and Enzyme Production*; Barredo, JL, Ed.; Research Signpost: Trivendrum, India, 2003. pp. 1–23.

Perez-Matos, AE; Rosado, W; Govind, NS. (2007) Bacterial diversity associated with the Caribbean tunicate *Ecteinascidia turbinata*. *Antonie van Leeuwenhoek*, *92*, 155–164.

Ravindra, J., Thilina, S. (2010): General ecology and habitat selectivity of fresh water fishes of the Rawan Oya, Kandy, Sri Lanka. Sabaragamuwa University Journal, 9, 11-43.

Rheinheimer, G. *Aquatic microbiology*; Wiley: Chichester; New York, NY, USA, 1992.

Ross, N., Islam, M., Thilsted, S. H. (2003): Small indigenous fish species in Bangladesh: Contribution to vitamin A, calcium and iron intakes. Journal of Nutrition, 133, 4021-4026.

Santiago-Vazquez, LZ., Bruck, TB., Bruck, WM., Duque-Alarcon, AP., McCarthy, PJ., Kerr, RG. (2007). The diversity of the bacterial communities associated with the azooxanthellate hexacoral *Cirrhipathes lutkeni*. *ISME J.*, 1: 654–659.

Ulfat Jan, Mustafa Shah, Tahila Manzoor and Showkat Ahmad Ganie. Variations of Protein Content in the Muscle of Fish *Schizothorax niger*. American-Eurasian Journal of Scientific Research 2012; 7 (1): 01-04.

Watve, MG., Tickoo, R., Jog, MM., Bhole, BD. (2001). How many antibiotics are produced by the genus *Streptomyces*. *Arch Microbiol.*, *176*, 386–390.

How to cite this article:

Deivasigamani, B., Vasuki Subramanian and Sundaresan, A. 2017. Identification of Protein from Muscle Tissue of Marine Finfish. *Int.J.Curr.Microbiol.App.Sci.* 6(4): 159-167.
doi: http://dx.doi.org/10.20546/ijcmas.2017.604.018