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Research Article

COLLECTION, MOLECULAR IDENTIFICATION AND ANTIBACTERIAL ACTIVITY OF MARINE FISH *CHELONODON PATOCA* FROM VISAKHAPATNAM COAST

[1]*Maheswari, D. and [2]Jagadish Naik, M.

[*1]Department of Zoology, Sri Gurajada Apparao Government Degree College, Yalamanchili, 531 055, Andhra Pradesh
[2]Department of Zoology, Acharya Nagarjuna University, Guntur - 522 510, Andhra Pradesh

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ABSTRACT

Terrestrial environment have relatively exhausted its resource for the pharmaceutical purpose. Recent decades, Marine ecosystem has been drawn attention of many scientists due to its biological and chemical diversity. A marine fishes was collected during from Tenneti beach (Long: 83°20' 59.94" E; Lat: 17° 44' 48.36" N) have been situated on the North East coast of Andhra Pradesh. And the COI region of the collected fish was amplified and sequenced, the BLAST results revealed that the organism was *Chelonodon patoca*. Protein, Lipids and compounds (methanolic) were extracted from skin, liver and ovaries. The antibacterial results revealed that protein extract of skin performed better antibacterial activity among the other extras.

Keywords: *Chelonodon pataco,* Antibacterial activity, Methanolic extracts, DNA barcoding.

INTRODUCTION

Marine ecosystem has drawn attention of many scientists due to its biological and chemical diversity (Chew *et al*., 2008). The number of natural products isolated from marine organisms increases rapidly and now exceeds with hundreds of new compounds being discovered every year (Chew *et al*., 2008; Proksch *et al*., 2012). Great progress has recently been made in the application of bioactive molecules isolated from marine organisms such as sponges, jellyfish, sea-anemones, shellfish (Blue-Biotechnology) representing an important resource useful to the health, food and processing or preservation industries (Salamone *et al*., 2012).

Marine bioresources produce a great variety of specific and potent bioactive molecules including natural organic compounds such as fatty acids, polysaccharides, polyether, peptides, proteins, enzymes and lectins. Proteins from marine sources show promise as functional ingredients in foods because they possess numerous important and unique properties such as film and foaming capacity, gel forming ability and antimicrobial activity (Rasmussen & Morrissey, 2007). Bioactive peptides usually contain 3-20 amino acid residues and their activities are based on their amino acid composition and sequence (Kim & Wijesekara, 2010). Many class of bioactive compounds exhibiting antitumour, antileukemia, antibacterial and antiviral activity have reported worldwide (Pettit *et al*., 1991). So far approximately 7000 natural products have been reported from the marine organisms (Venkataraman *et al*., 2005). Among them, fishes are the largest classes of vertebrates. Due to the aquatic environment, fishes have some unique characteristics (Ellis, 2001; Plouffe *et al*., 2005). Fishes are evolving with innate immune response to protect themselves against the infection.

Puffer fishes are famous for their powerful toxin, making the fish inedible except prepared by skilled fugu chefs. Puffer fishes possess a toxin called tetradotoxin (ttx), for its presence in fishes the order was named as tetraodonti formes, which including puffer fishes and porcupine fishes and is then concentrated in the skin and liver of the fish (Wu *et al*., 2005). Puffer fish are initially considering the only animals that contain TTX, later this compound has been reported from a taxonomically diverse range of

*Corresponding Author: Dr. Maheswari, D., Department of Zoology, Sri Gurajada Apparao Government Degree College, Yalamanchili, 531 055, Email: maheswari.mamu@gmail.com

marine and terrestrial organisms from 14 different phyla (González Sainz *et al*., 2013).

Chelonodon patoca, belonging to the family Tetraodontidae, is the puffer fish species reported in the freshwater habitats of the Western Ghats, India (Hora & Law, 1941). *C. patoca* (Hamilton, 1822) by original designation; estuaries of the Ganges (West Bengal, India). Antibacterial proteins are assumed to form ion channels in bacterial membrane and kill both gram positive and gram negative bacteria (Ebran *et al*., 2000). Knouft *et al*. (2003) has reported endogenous peptides with antimicrobial activity from fish mainly from the skin and its secretions. In India, studies on the antibacterial activity of puffer fish are very limited. The aim of the present study is to evaluate the antibacterial activity of skin, liver and ovaries of *C. patoca* collected from Visakhapatnam coast.

MATERIALS AND METHODS

Sample Collection

Healthy marine fishes *C. patoca* was collected during May 2016 from Tenneti beach (Long: 83°20' 59.94" E; Lat: 17° 44' 48.36" N) have been situated on the North East coast of Andhra Pradesh, adjoining the Bay of Bengal, Visakhapatnam, India (Figure 1). Using hand nets, trammel nets, cast nets. The collected samples were aseptically placed in ice filled containers and transported to the laboratory. First sample washed under tap water and then with distilled water. Each sample was filleted - muscle tissue was separated from the skin with a knife. Three replicate samples were prepared. The dissected samples were stored at -20°C for further analysis.

DNA extraction from fish muscle

Fish muscle tissue was suspended in 500 µl of buffer (20 mM Tris-Cl, 5 mM EDTA, 400 mM NaCl, 1% (w/v) SDS, 400 µg/ml Proteinase K), homogenized overnight at 55°C in a shaking incubator with oscillation of 200 rpm. Equal volume of Phenol: Chlorophorm: Isoamyl Alcohol (25:24:1) was added to the dissolved liquid, placed in shaking incubator at room temperature for 30 min and centrifuged at 13000 rpm for 5 min. Upper aqueous layer was transferred to a new microcentrifuge and equal volume of isopropanol was added and centrifuged again at 8000 rpm for 15 minutes. The isopropanol was removed and the remaining was washed with 70% ethanol. Air dried DNA was resuspended in nuclease free water.

The COI standard barcoding region (652bp) was amplified for two fish samples using a pair of degenerate fish primers (Fish-Uni F5'CA CGACGT T G T A A A A C G A C A C Y A A I C A Y A A A G A Y A TIGGCAC-3'; Fish-uni-R- GGATAACAATTTCACAC A GGACITCAG GGTGWCC AARAAYCARAA-3') as well as a primer cocktail previously described. Each amplification reaction contained 2µl DNA template, 17.5µl molecular biology grade water, 2.5µl 10X reaction buffer, 1µl MgCl2 (50µM), 0.5µl dNTPs mix (10 mM), 0.5µl forward primer (10µM), 0.5µl reverse primer (10µM), and 0.5µl Invitrogen's Platinum Taq polymerase (5U/µl) in a total volume of 25µl. The PCR conditions were initiated with a heated lid at 95 °C for 5min, followed by a total of 35 cycles of 94 °C for 40 S, 51 °C for 1min, and 72 °C for 30 S, and a final extension at 72 °C for 5min, and hold at 4 °C. PCR reactions were carried out using Mastercycler ep gradient S (Eppendorf, Mississauga, ON, Canada) thermal cyclers. PCR success was verified by 1.5% agarose gel electrophoresis. A DNA template negative control reaction was included in all experiments to test for contamination. Two microliters of each amplicon were subsequently used directly for bi-directional Sanger sequencing using Applied Biosystems's BigDye Terminator chemistry V3.1. Identification of the tested samples was conducted using BLAST in GenBank and a local barcode library for selected taxa with a minimum BLAST cut off of 98% identity for a top match.

Extractions

Three fish parts (skin, liver and ovary) were separated, washed and allow them for extraction in the below methods.

Methanolic extraction

Hundred gm of fish parts were weighed and transferred into conical flask. Then 1000ml methanol was added. The conical flask was closed by foil paper and put in dark place at maximum 7 days. The crude methanol extracts were then filtered by passing the extracts through filter paper. After filtration, the extracts were placed in rotary vacuum evaporator to concentrate.

Protein extraction

Ferreira *et al*., 2002) method was used to extract the total protein by using poly vinyl pyrrolidone (PVP). 5 gm of skin and tissue samples of *C. patoca* and was homogenised separately in 50 mM sodium phosphate buffer containing 10% insoluble PVP and incubated at 40°C for overnight. Then the homogenates were centrifuged at 14000 rpm for 20 min at 4°C. The supernatant was collected and stored at -20°C for further protein research analysis.

Lipid extraction

Bligh & Dyer, (1959) method was used for the extraction of total lipids from the skin tissues of *C. patoca.* 1 gm skin from each sample was homogenised separately with3.75 ml methanol: chloroform (2:1V/V) and 1 ml of 1 mM EDTA in 0.15 Macetic acid was added. Homogenate was transferred to new glass tube and the homogenizer rinsed with 1.25 ml of chloroform and transferred to the tube, finally1.25 ml of 0.88% KCl was added and centrifuged at 3000 rpm for 2 min. The lower phase which contains lipid

was transferred to new tube. The lipid was stored in a refrigerator at 4-6°C for further analysis.

Antibacterial activity Microorganisms

The below said micro organisms were obtained from IMTECH Chandigarh. The organisms were, *Bacillus subtilis, Salmonella, S. aureus* and *V. cholera.*

Agar-well diffusion testing

Antibacterial activities of the compounds investigated were first evaluated by agar-well diffusion method. The standardized cultures of test bacteria were first evenly spread onto the surface of Mueller Hinton Agar plates using sterile cotton swabs. Five wells (6 mm diameter) were made in each plate with sterile cork borer. Fifty microliters of each of the compound and positive control was added in wells. Gentamicin (200 µg/mL), vancomycin (1 µg/mL) were used as reference antibiotics. Diffusion of compounds, antibiotics and DMSO were allowed at room temperature for 1 h. All of the plates were then covered with lids and incubated at 37 ºC for 24 h. After incubation, plates were observed for zone of bacterial growth inhibition. The size of inhibition zones was measured and antimicrobial activity of the compounds was expressed in terms of the average diameter of inhibition zone in millimeters. Those compounds that were unable to exhibit inhibition zone (inhibition zone diameter less than 6 mm) were considered non-active. Each compound was tested in triplicate with two independent experiments and mean values of inhibition zone diameters were taken.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) values of the plant extracts were determined by microbroth dilution method. The test bacteria from the stock cultures were inoculated in MHB and incubated at 37°C under stirring for 24 h. The bacterial suspensions were then diluted with fresh MHB to achieve the turbidity equivalent to 0.5 McFarland standard. Different dilutions to get the final concentration ranging from 49 to 25000 µg/mL for the plant extract and 1 to 256 µg/mL for the reference antibiotics (gentamicin and vancomycin) were prepared in MHB directly in the wells of 96-well plates in a final volume of 200 µL. In each of these dilutions, 100 µL was of bacterial suspension (approx. 1.5 x 10^6 CFU/mL). The highest percentage of DMSO being used in the wells was 2.5 % and was not found to inhibit the growth of test bacteria. The wells containing 100 µL MHB, 100 µL bacterial inoculum and DMSO at a final concentration of 2.5 % served as negative control. The plates were covered with sterile plate sealer and were agitated to mix the contents of the wells by using shaker. Then the plates were incubated at 37oC for 18 h. After incubation, 40 µL of 0.2 mg/ml p-iodonitrotetrazolium violet indicator solution was added to every well in order to assess the bacterial growth. This indicator solution changes its color from colorless to red in the presence of bacterial growth and the degree of redness is a good indicator of inhibitory effect of extract or antibiotic on bacterial growth. After addition of this indicator solution, the plate was incubated for an additional 30 minutes. The MIC was defined as the lowest concentration of extract or antibiotic in which there was no visible growth of a test bacterium.

RESULT AND DISCUSSION

Total 621 base pairs sequence was received; the sequence was subjected to NCBI Blast analysis to know the similar sequences. 97.91% similarity was found for the *C. patoca* voucher BIF1422 cytochrome oxidase subunit 1 (COI) gene. And in the UPGMA tree analysis also the query sequences was present in the same group with *C. patoca* (Figure 2). And the physical characters also similar to that organism.

> *Chelonodon patoca* voucher

AGTGGGGACCGCCCTAAGTCTCCTCATTCGGGCTG
AACTCTGCCAACCGGGTGCGCTCCTAGGCGACGAT
CAGATTTACAATGTGATCGTCACAGCCCATGCATT
TGTAATAATCTTCTTTATAGTAATACCAGTCATGA
TCGGCGGGTTCGGAAACTGACTAGTCCCTCTTATA
ATTGGGGCCCCTGACATAGCATTCCCTCGGATGAA
CAATATAAGTTTCTGACTATTGCCCCCTTCCTTCCT
TCTTCTCCTAGCTTCTTCCGGCGTAGAAGCCGGGG
CCGGCACAGGCTGAACCGTCTACCCTCCGCTAGCA
GGCAACCTAGCCCACGCAGGCGCATCCGTCGACCT
CACCATCTTCTCCCTCCACCTAGCAGGTGTTTCATC
AATCCTAGGCGCTATTAACTTCATCACCACAATCA
TTAACATAAAACCCCCTGCCATCTCCCAGTACCAA
ACGCCTCTCTTCGTATGGGCCGTTCTAATCACCGC
CATCCTTCTTCTTCTATCCCTACCCGTCCTCGCAGC
AGGCATTACAATGCTCCTTACGGACCGAAACCTAA
ACACCACCTTCTTCGACCCCGCAGGCGGAGGAGA
CCCCATCCTCTACCAGCACTTATT.

Antibacterial activity of *C. patoca*

We have done antibacterial activity using the three part of the COI standard i.e. skin, liver and ovaries. From each part protein, lipid and methanolic compound extracts were separated and antibacterial activity was done for them. In *C. patoca* three samples studied were showed antibacterial activity against the tested four organisms. All the three samples showed similar activity. In the four bacteria studied *S. aureus* and *Salmonella* were more sensitive than the remaining organisms, they showed 19mm and 20mm zone of inhibitions at 10mg concentration respectively. *V. Cholera* and *B. subtilis* were in the next place of showing inhibition activity. In case of gram positive micro organisms consistently protein extract was found to be better antibacterial activity. In *B. subtilis* skin protein showed 15 mm of zone of inhibition and in case of *S. aureus,* skin protein showed 19 mm zone of inhibition at 10 mg concentration. Two gram native organisms

Table 1. Zone of inhibition antibacterial activity of extracts of *Chelonodon patoca*.

		Zone of inhibitions in mm							
		Gram positive				Gram Negative			
		Bacillus subtilis		*S. aureus*		*Salmonella*		*V. cholerae*	
		10 mg	5 mg	10 mg	5 mg	10 mg	5 mg	10 mg	5 mg
Skin	Protein	15	12	19	15	20	18	17	10
	Methanol	10	0	14	10	11	10	9	0
	lipids	12	9	20	17	18	15	14	9
Liver	Protein	11	8	15	12	11	8	0	0
	Methanol	7	0	8	0	0	0	0	0
	Lipids	10	7	12	10	10	0	0	0
Ovaries	Protein	8	7	9	8	12	10	13	12
	Methanol	0	0	0	0	9	0	8	0
	Lipids	8	0	0	0	10	9	12	10
Gentamicin (200 µg/mL)		21		19		17		19	

Table 2. Minimal Inhibition Concentration (MIC) values of crude extracts.

		MIC (µg/ml) Gram positive			
		Bacillus subtilis	*S. aureus*	*Salmonella*	*V. cholerae*
Skin	Protein	355	320	230	355
	Methanol	650	390	620	820
	Lipid	560	320	355	420
Liver	Protein	620	355	620	--
	Methanol	1250	755	--	--
	Lipid	640	560	755	--
Ovaries	Protein	925	720	640	420
	Lipid	--	--	820	860
	Methanol	925	--	755	550

Figure 1. C*helonodon patoca* collected sample.

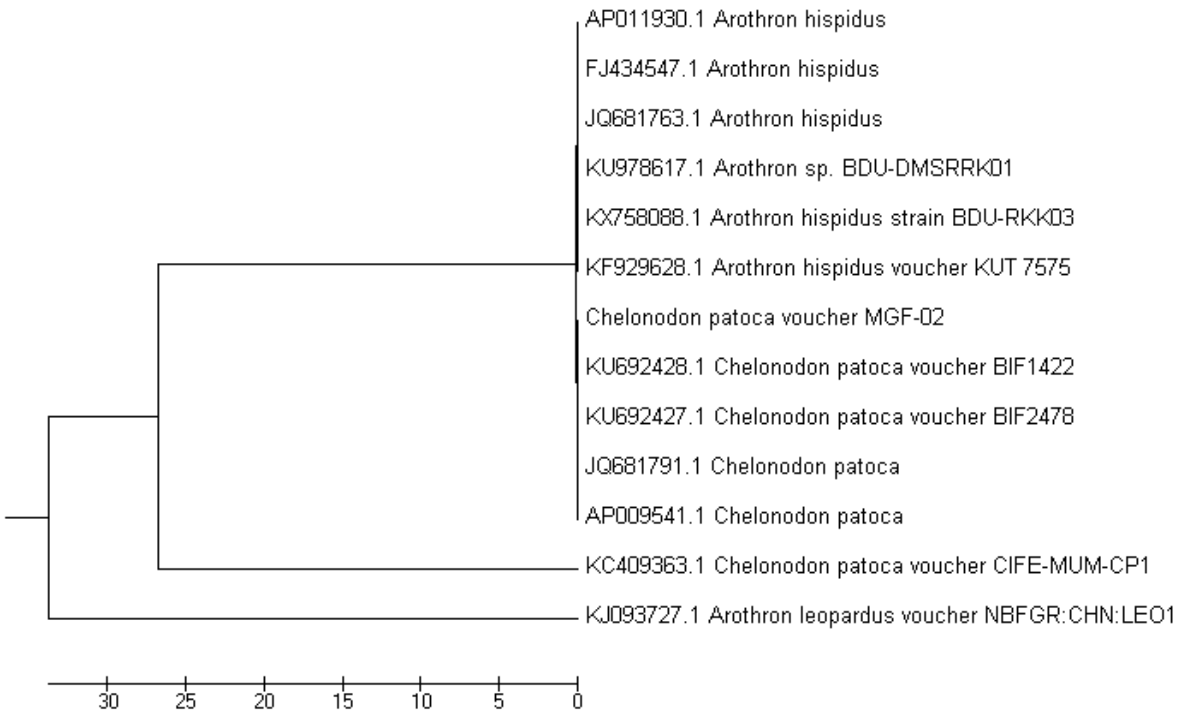
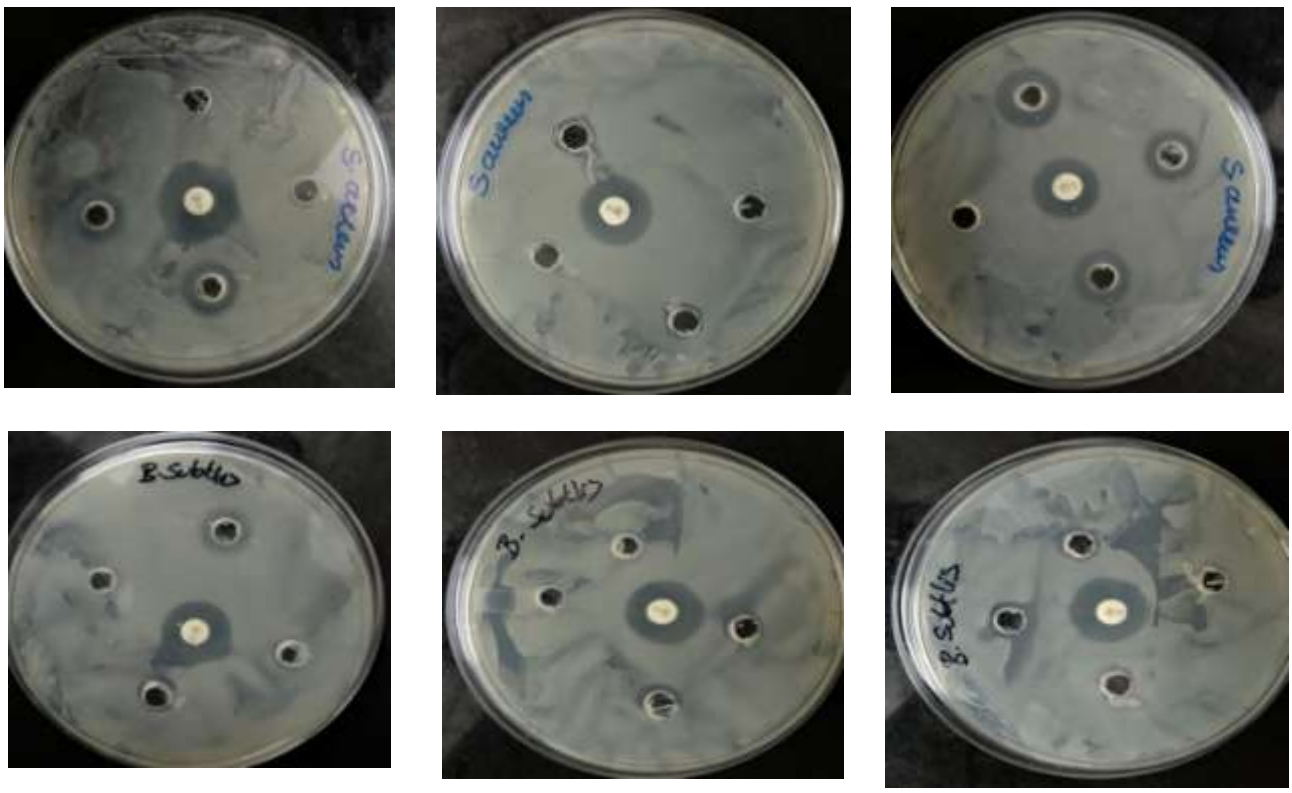


Figure 2. Evolutionary relationships of taxa using the UPGMA method.



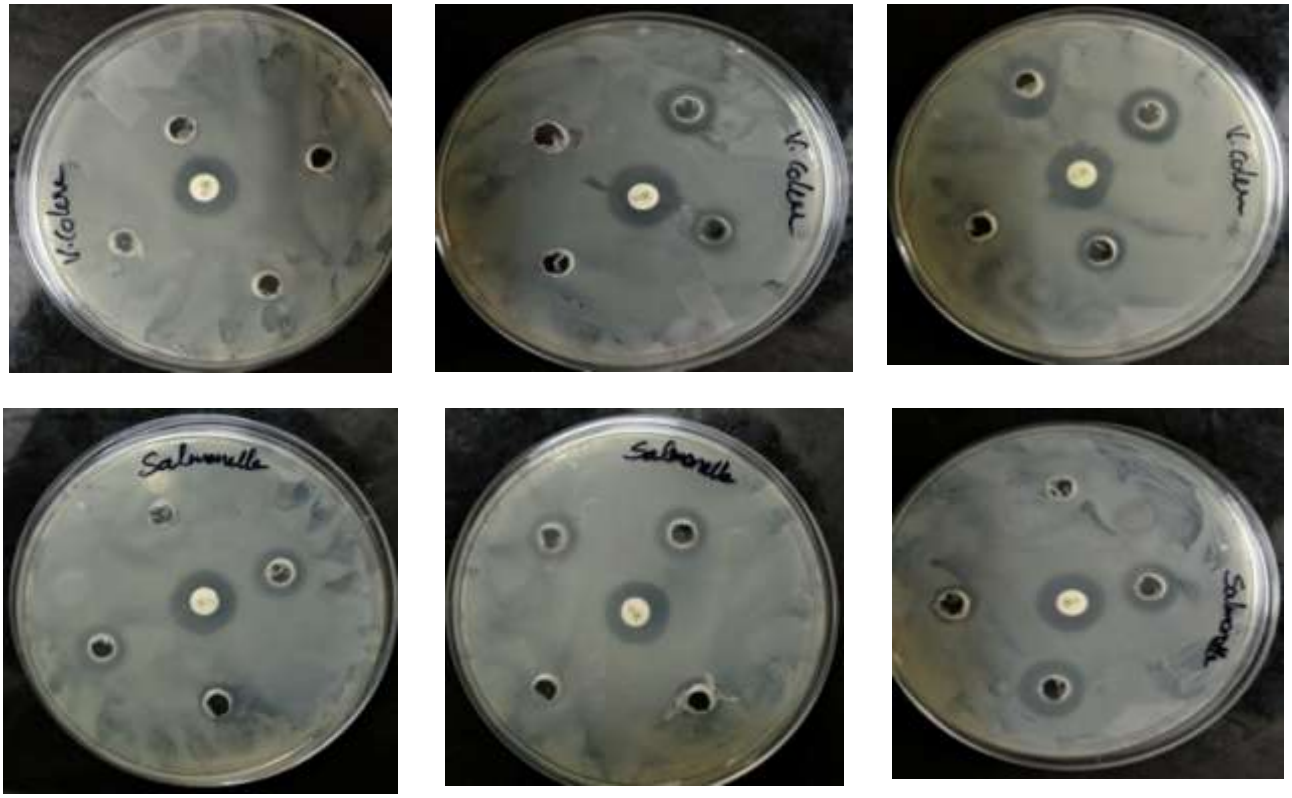


Figure 3. Anti-bacterial plates showing zone of clearance with *Chelonodon patoca* extracts against pathogenic bacteria. First row liver extracts, second row ovary extracts and third row skin extracts.

were used for the study, among them skin protein showed maximum 20 mm zone of inhibition against *Salmonella* bacteria. Same like skin protein and ovaries protein extracts showed maximum 17mm and 13 mm zone of inhibitions against *V. cholera* (Table 1, Figure 3).Minimal Inhibition Concentration (MIC) values were varied with each extract. Lower MIC values (230 µg/ml) were found for Skin protein extract in case of *Salmonella* organisms. On the other hand 320 µg/ml MIC was found for the skin protein and skin lipid extracts against *S. aureus* (Table - 2). Based on the complete results revealed that protein extract showed maximum bacterial inhibitions among them skin protein extract performed maximum activity. In 1950s first attempt of antimicrobial activities for marine organisms was initiated (Shaw *et al*., 1976). Many of these organisms have antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to complete with classical antimicrobial activity against microorganisms (Kumaravel *et al*., 2010). Fish is the earliest and the largest class vertebrate with its innate immune system being considered as the predominant mechanism for host defense (Indumathi *et al*., 2016) which includes excretion of antimicrobial peptides, polypeptides, non-classical complement activation, cytokine release, inflammation and phagocytosis (Fernandes & Smith, 2002; Magnadóttir, 2006). On the other hand, fishes hold the credit of possessing rich protein sources. These marine proteins are not only correlated to the intact proteins, but also to the possibility of generating bioactive peptides (Perez, 1990). In recent years, different toxins derived from marine sources have been identified as having potential antimicrobial activities. Precisely, fishes evolved several innate immune mechanisms to defend microbial infection (Indumathi *et al*., 2016). On the other hand, fishes hold the credit of possessing rich protein sources. These marine proteins are not only correlated to the intact proteins, but also to the possibility of generating bioactive peptides (Ngo *et al*., 2011). Most of reported antimicrobial peptides typically have strong antimicrobial activity against a wide range of Gram-positive bacteria but very weak or no activity against Gram-negative bacteria, like mitomycin (Mitta *et al*., 2000).

CONCLUSION

The collected fish sample was confirms as *C. patoca* by doing the sequencing of COI region. And the antibacterial evaluation revealed that protein extracts were proved to be higher antibacterial compounds. Skin extracts were having the better antibacterial compounds among the three tissues studied.

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REFERENCES

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian journal of Biochemistry and Physiology, 37*(8), 911-917.

Chew, Y. L., Lim, Y. Y., Omar, M., & Khoo, K. S. (2008). Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT-Food Science and Technology, 41*(6), 1067-1072.

Ebran, N., Julien, S., Orange, N., Auperin, B., & Molle, G. (2000). Isolation and characterization of novel glycoproteins from fish epidermal mucus: correlation between their pore-forming properties and their antibacterial activities. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1467*(2), 271-280.

Ellis, A. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology, 25*(8-9), 827-839.

Fernandes, J. M., & Smith, V. J. (2002). A novel antimicrobial function for a ribosomal peptide from rainbow trout skin. *Biochemical and Biophysical Research Communications, 296*(1), 167-171.

Ferreira, R. R., Fornazier, R. F., Vitória, A. P., Lea, P. J., & Azevedo, R. A. (2002). Changes in antioxidant enzyme activities in soybean under cadmium stress. *Journal of Plant Nutrition, 25*(2), 327-342.

González-Sainz, C., Ruiz-Redondo, A., Garate-Maidagan, D., & Iriarte-Avilés, E. (2013). Not only Chauvet: dating Aurignacian rock art in Altxerri B Cave (northern Spain). *Journal of Human Evolution, 65*(4), 457-464.

Hamilton, F. (1822). *An account of the fishes found in the river Ganges and its branches*: Bishen Singh Mahendra Pal Singh. p-226.

Hora, S., & Law, N. (1941). The freshwater fishes of Travancore. *Records of Indian Museum, 43*, 233-256.

Indumathi, S., Manigandan, V., & Khora, S.(2016). Antimicrobial and larvicidal activities of the tissue extracts of oblong blowfish (*Takifugu oblongus*) from South-East Coast of India. *International Journal of Toxicological and Pharmacological Research*, 8(5), 312-319 .

Kim, S. K., & Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods, 2*(1), 1-9.

Knouft, J. H., Page, L. M., & Plewa, M. J. (2003). Antimicrobial egg cleaning by the fringed darter (Perciformes: Percidae: *Etheostoma crossopterum*): implications of a novel component of parental care in fishes. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 270*(1531), 2405-2411.

Kumaravel, K., Ravichandran, S., Balasubramanian, T., Siva Subramanian, K., & Bilal, A. (2010). Antimicrobial effect of five seahorse species from the Indian coast. *British Journal of Pharmacology and Toxicology, 1*(2), 62-66.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish and Shellfish Immunology, 20*(2), 137-151.

Mitta, G., Vandenbulcke, F., & Roch, P. (2000). Original involvement of antimicrobial peptides in mussel innate immunity. *FEBS letters, 486*(3), 185-190.

Ngo, D. H., Wijesekara, I., Vo, T. S., Van Ta, Q., & Kim, S. K. (2011). Marine food-derived functional ingredients as potential antioxidants in the food industry: An overview. *Food Research International, 44*(2), 523-529.

Perez, C. (1990). Antibiotic assay by agar-well diffusion method. *Acta Biological Medical Expriment, 15*, 113-115.

Pettit, G. R., Herald, C. L., Boyd, M. R., Leet, J. E., Dufresne, C., Doubek, D. L., Rutzler, K. C. (1991). Antineoplastic agents. 219. Isolation and structure of the cell growth inhibitory constituents from the western Pacific marine sponge Axinella sp. *Journal of Medicinal Chemistry, 34*(11), 3339-3340.

Plouffe, D. A., Hanington, P. C., Walsh, J. G., Wilson, E. C., & Belosevic, M. (2005). Comparison of select innate immune mechanisms of fish and mammals. *Xenotransplantation, 12*(4), 266-277.

Proksch, P., Ebel, R., Edrada, R., Wray, V., & Steube, K. (2012). Invertebrates and Associated Fungi. *Sponges* (*Porifera*), *37*, 117.

Rasmussen, R. S., & Morrissey, M. T. (2007). Marine biotechnology for production of food ingredients. *Advances in Food and Nutrition Research, 52*, 237-292.

Salamone, M., Cuttitta, A., Seidita, G., Mazzola, S., Bertuzzi, F., Ricordi, C., & Ghersi, G. (2012). Characterization of collagenolytic/proteolytic marine enzymes. *Chemical Engineering, 27*, 1-6.

Shaw, P., McClure, W., Van Blaricom, G., Sims, J., Fenical, W., & Rude, J. (1976). Antimicrobial activities from marine organisms. *Fooddrugs from the sea. Washington DC: Marine Technology Society*, 55-60.

Venkataraman, C., Habib, G., Eiguren-Fernandez, A., Miguel, A., & Friedlander, S. (2005). Residential biofuels in South Asia: carbonaceous aerosol emissions and climate impacts. *Science, 307*(5714), 1454-1456.

Wu, Z., Yang, Y., Xie, L., Xia, G., Hu, J., Wang, S., & Zhang, R. (2005). Toxicity and distribution of tetrodotoxin-producing bacteria in puffer fish Fugu rubripes collected from the Bohai Sea of China. *Toxicon, 46*(4), 471-476.