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Purification of Protein from Marine Edible Oyster *Crassostrea madrasensis* for Bactericidal Potency

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Nowadays, the pharmaceutical market is growing rapidly and continuously in worldwide but, still the demand for new drug discovery is encouraged. Because, the growth of numbers drug resistant infectious disease and more upcoming disorders to human and animals. In general, the marine animals especially mollusks and their compounds constitute a practically unlimited resource of new active substances. Hence, the present study was carried out to determine the bactericidal activity of *Crassostrea madrasensis* protein against human pathogens. The edible Oyster *Crassostrea madrasensis* was collected from Rayapuram lnading centre, Tamil Nadu, India. Immediately it was extracted by using phosphate buffer at three different pH (4, 7 and 9) and all the extracts were screened against six different human pathogens such as *Vibrio cholerae*, *V. parahaemolyticus*, *Salmonella* sp, *Shigella* sp, *Streptococcus* sp and *Staphylococcus* sp by agar well diffusion assay. After 24 hrs of incubation the maximum inhibitory effect was observed against *Vibrio parahaemolyticus*, *Streptococcus* sp and *Staphylococcus* sp and the minimum inhibitory effect was observed against *Vibrio cholerae*, *Salmonella* sp and *Shigella* sp respectively. Whereas checking the minimal inhibitory concentration (MIC), the crude protein extract of *Crassostrea madrasensis* was inhibited the bacterial strains with the minimum inhibitory concentration of not less than 0.1ml (100¼l). The molecular weight of the crude protein was found from 12.2 to 74.2 kDa and the total protein content of phosphate buffer crude extract of *Crassostrea madrasensis* was found to be 312 ¼g/ mg. From, the results, the work has suggested to use this commercially available and protein rich (bactericidal) oyster in therapeutics for the development of novel antibiotics against multiple drug resistance (MDR) pathogenic microbes.

Key words: *Crassostrea madrasensis*, Human pathogens, Antibacterial activity, Bactericidal activity.

The growth of many drug resistant infectious disease and more upcoming disorders to human and animals are major problem in world wide. Antimicrobial protein (peptides) is become as new antibacterial substance because of their bioactivity against resistance bacteria. The terrestrial resources have been greatly explored. Nowadays, the researchers are expecting the lead molecules and compounds from the new resources especially. The Ocean covered more than 70% of the earth surface represent an enormous resource and from the past three to four decades many efforts have been committed for isolating various biologically active novel compounds from marine bio sources due to their huge biodiversity and

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which offers a potential chemicals which can be useful for finding new bioactive compounds with greater effectiveness and specificity against human and animal pathogens [1]. There are, more than 12,000 natural products have been isolated from Marine algae, sponges, coelenterates, ascidians, echinoderms and bryozoans [2]. Molluscs are a common prospect resource for the discovery of novel compounds for isolating bio active compounds to the pharmaceutical industry because most of the marine animals have lack of physical defenses, they produce toxic chemicals to protect themselves in a very hostile environment and still now most of them are unexplored [3,4]. Some studies have reported that, the bioactivity of the mollusks like *Aplysia* sp [5], *Phyllidae* sp [6], bivalves [7], gastropods [8], and their egg masses [9]. Moreover, marine invertebrates are known to depend on innate immune mechanisms by interacting cellular and humoral components to protect against pathogens for their safe[10]. The *Crassostrea madrasensis* is one of the edible and commercially available species and the metabolites or bioactive compounds are still unexplored [11]. Thus the present study was carried out to determine the bactericidal effect of commercially available and edible oyster *Crassostrea madrasensis* extract against six different human pathogens.

MATERIALS AND METHODS

Collection and Identification of *Crassostrea madrasensis*

The edible Oyster *Crassostrea madrasensis* was collected from Rayapuram landing centre, Tamil Nadu, India. The collected animals were identified by using standard manuals[12].

Extraction of Bactericidal peptides

The edible Oyster *Crassostrea madrasensis* was collected and transferred to the laboratory and washed with distilled water and the flesh samples were taken by breaking the shells. The peptides were prepared from the whole body tissue by phosphate buffer saline at three different pH (4, 7 and 9) by standard homogenization procedure. The homogenized mixtures were centrifuged at 4°C in 7500 rpm for 30 min. The supernatant was obtained, Freeze dried and stored at -20°C.

The lyophilized crude extract was dissolved in 9.5 ml of phosphate buffer saline (PBS) at three different pH to obtain the partially purified protein by 85% ammonium sulfate precipitation and it was dialyzed[13]. The dialyzed solution was freeze dried and stored at -20°C. A stock solution of 2 mg/ml of lyophilized crude protein extract in sterilized PBS at three different pH(4, 7 and 9) was prepared for the further test[14].

Bactericidal activity

The bactericidal potency of the crude protein extract of *Crassostrea madrasensis* was evaluated by adding 100¼l of each extract (water and phosphate buffer) against six different human pathogens such as *Vibrio cholerae*, *V. parahaemolyticus*, *Salmonella* sp, *Shigella* sp *Streptococcus* sp and *Staphylococcus* sp by agar well diffusion assay. After the 24 hrs incubation, the zone of inhibition (ZOI) around the wells was measured. The assay was repeated in triplicate and the averages of the three were given as results [15].

MIC and MBC determination

The minimal inhibitory concentration (MIC) of the crude extract of *Crassostrea madrasensis* was determined by broth tube dilution assay. The *Crassostrea madrasensis* crude extract was prepared at various concentrations from 0.1ml to 0.5ml were determined for inhibitory level against all the human bacterial pathogens. The minimal inhibitory concentration (MIC) tubes were further carried out for Minimal Bactericidal concentration (MBC) evaluation using standard protocols. After 24 hrs of incubation period the loop full of cultures from the MIC and control tubes were transferred to the nutrient agar plates and the growth was monitored [16].

SDS PAGE Analysis

The proteins in the crude extract of *Crassostrea madrasensis* were purified and the molecular weight was confirmed by SDS PAGE analysis[17].

Estimation of protein concentration

The total protein concentration in the crude extract of *Crassostrea madrasensis* was estimated by the Lowry's method using BSA as standard[18].

RESULTS AND DISCUSSION

In general, the marine invertebrates such as cephalopods, gastropods, bivalves secrete or emit some substances which have a role in the chemical defenses and act against their predators. Some studies also proven that the compounds isolated from mollusks have exhibiting several activities against human and animal pathogens[19]. It is estimated, there are more than thousand new compounds has been categorized from marine invertebrates such as peptides, terpenes, polypropionates, nitrogenous compounds, polypeptides, macrolides, prostaglandins and fatty acid products, sterols and diverse compounds[20]. Among the marine invertebrates the bivalves possess several types of defense molecules including agglutinins and glycoproteins which have bactericidal activities[21]. In this present study, the edible Oyster *Crassostrea madrasensis* was collected from Rayapuram landing centre of Tamil Nadu, India (Fig 1). Immediately it was extracted by using phosphate buffer at three different pH (4, 7 and 9) and all the extracts were screened against all the six different human pathogens such as Vibrio *cholerae*, *V. parahaemolyticus*, *Salmonella* sp, *Shigella* sp, *Streptococcus* sp and *Staphylococcus* sp by agar well diffusion assay and the zone around the wells were measured after incubation of 24 hrs (Table 1).

Table 1. Antimicrobial activity of *Crassostrea madrasensis* extract against human pathogens

S. No	Human pathogens	Zone of Inhibition (mm)		
		pH (4)	pH (7)	pH (9)
1	*Vibrio cholerae*	+	++	++
2	*V. parahaemolyticus*	++	+++	+
3	*Salmonella* sp	+	++	++
4	*Shigella* sp	+	++	++
5	*Staphylococcus* sp	++	+++	++
6	*Streptococcus* sp	++	+++	+

+ : 8mm ++: 12mm +++: 14mm -: No Inhibition

Moreover, in all the tested human pathogenic bacteria were mostly inhibited by the crude protein extract pH 7 of *Crassostrea madrasensis* and the maximum inhibitory effect of 14mm was observed against *Vibrio parahaemolyticus*, *Streptococcus* sp and *Staphylococcus* sp and the minimum inhibitory effect of 8mm was observed against *Vibrio cholerae*, *Salmonella* sp and *Shigella* sp respectively (Fig 2). Similar results were also observed by previous studies[22]. In their study they have reported that the edible bivalves *Perna viridis* and *M. casta* have the ability to inhibit growth of pathogenic bacteria *Staphylococcus aureus* and *Salmonella enteridis*, which cause food borne illness[23]. Three different extracts of both *M. meretrix* and *M. casta* species against some pathogens, both the extracts have showed highest antibacterial activities against *B.substillus*, *K.pneumonia* and *P.fluroscence* respectively [23].

Whereas checking the minimal inhibitory concentration (MIC) of the crude extract of *Crassostrea madrasensis* against all the human pathogenic bacteria at different dilutions (such as, 0.1, 0.2, 0.3, 0.4 and 0.5ml) by broth tube dilution assay. The extract has inhibited the bacterial strains with the minimum inhibitory concentration of not less than 0.1ml (100¼l) of the extract. The pathogenic bacterial strains such as *Vibrio parahaemolyticus*, *Streptococcus* sp and *Staphylococcus* sp were inhibited at 200¼l and remaining pathogenic bacterial strains such as, *Vibrio cholerae*, *Salmonella* sp and *Shigella* sp were inhibited at 300 ¼l of *Crassostrea madrasensis* extract. Moreover the extracts also just inhibited the growth of many pathogenic bacteria at higher concentrations but not killed. The inhibitory and bactericidal concentration (MBC) remains same for extract of *Crassostrea madrasensis* against *Vibrio cholerae*, *V. parahaemolyticus* and *Salmonella* sp

(Table:1). Similar results were observed when inhibiting the growth of *Proteus vulgaris, Klebsiella pneumonia and Salmonella typhi* by using the crude protein extracts of *Pitar erycina* and *Donax cuneatus*[15].

While analyzing the molecular weight of the crude protein extract of *Crassostrea madrasensis* using SDS-PAGE analysis with the marker range 14.4 to 97.4 kDa the results obtained with the separation of protein at 12.2, 15.1, 17.5, 27.1, 32.8, and 51.6 and 74.2 kDa (Fig 3). [1]Previous author have observed 5-6 bands ranging from 45 to 223 kDa from the extracts of *Meretrix meretrix* and *Meretrix casta*, similarly 35 kDa from *Perna canaliculus*[24], 9.7 kDa from *Perna viridis* [25] and 3.5 Kda to 200 Kda from *Donax cuneatus and Pitar erycina* [16]. The total protein (312 ¼g/ mg) in the extract of *Crassostrea madrasensis* was determined with the help of Lowry's method. The previous authors also reported, that the mantle and tissues of *Meretrix casta* has 190 ¼g mg^{-1} mL^{-1} protein, 5.76 ¼g mg^{-1} mL^{-1} carbohydrates and 0.15 ¼g $mg^{-1}mL^{-1}$ lipid respectively[22].The nutritional composition of three estuarine bivalve's *Perna viridis, Donax caneatus* and *Meretrix meretrix* also resulted[26]. In general, antimicrobial peptides (AMPs) are also act as major components of innate immune defence system in invertebrates, because the innate immunity is triggered immediately when the microbial infection occurs[27]. From the results, the study has suggested to use this antibacterial peptides from *Crassostrea madrasensis* for the development of novel antibiotics against to multiple drug resistance (MDR) pathogenic microbes.

Fig. 1. *Crassostrea madrasensis*

Fig. 2. Antibacterial activity of *Crassostrea madrasensis* extract against human pathogens

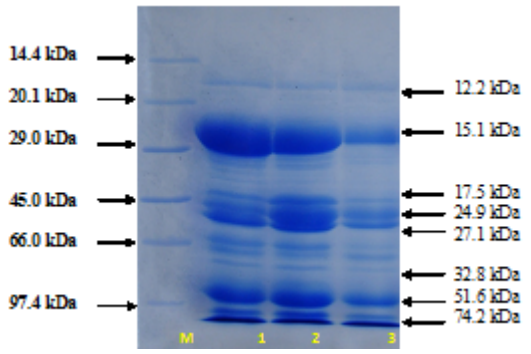

Fig. 3. Protein profile for the crude extract of *Crassostrea madrasensis* in SDS PAGE

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REFERENCES

1. Sugesh S and Mayavu P, Antimicrobial activities of two edible Bivalves *M.meretrix and M.casta, Pakistan Journal of Biological Sciences*, 2013; **16**(1): 38-43.
2. Constantino V, Fatturusso E, Meena M and Taglilatele-Scafati O, Chemical diversity of bioactive marine natural products: an illustrative study, *Current Medical Chemistry*, 2004; **11**: 1671- 1692.
3. Chellaram C, Gnanambal KME and Edward JKP, Antibacterial activity of the winged oyster *Pteria chinensis* (Pterioida: Pteridae), *Indian*

Journal of Maine Sciences, 2004; **33**(4): 369-372.

4. Kathiresan K, Nabeel MA and Manivannan S, Bioprospecting of marine organisms for novel bioactive compounds, *Journal of Science Transmission Environmental Technnovation*, 2008; **1**:107-120.
5. Stallard MO and Faulkner DJ, Marine natural products from mollusks, *Journal of Comparative Biochemistry and Physiology*, 1974; **49**:25-32.
6. Ilagedone MR, Barreson BJ and Schener PJ. Bioactive natural products, *Halvetica chemica acta*, 1999; **62**:2484-2486.
7. Jayaseeli AA, Prem Anand T and Murugan A, Antibacterial activity of four-bivalves from Gulf of Mannar, Phuket Marine Biological Center for Special Publication, 2001; **25**: 215-217.
8. Emerson Kagoo L and Ayyakkannau K, Bioactive compounds from *Chicoreus ramosus* antibacterial activity - In vivo, Phuket *Marine Biological Center for Special Publication*, 1992; **11**: 147-150.
9. Prem Anand T, Raja Ganapathy J and Patterson Edward JK, Antibacterial activity of marine molluscs from portnovo region, *Indian Journal of Marine Sciences*, 1997; **26**:206-208.
10. Tincu AJ and Taylor SW, Antimicrobial peptides from marine invertebrates, *Antimicrobial Agents and Chemotherapy*, 2004; **48**: 3645-3654.
11. Mallia JV, Muthiah P and Thomas PC, Growth of triploid oyster *Crassostrea madrasensis* (Preston), *Journal of Aquaculture Research*, 2006; **37**: 718-724.
12. Rao KS, Edible bivalves mussels and oysters. In: The Commercial Molluscs of India, Bulletin of the Central Marine Fishery Research Institute, 1974; **25**: 4-39.
13. Scopes RK. Protein purification. Principles and practice 2nd Edn, Springer-verlag, New York; 1987; 302-306.
14. Pakrashi A, Roy P and Datta U, antimicrobial effect of protein(s) isolated froma marine mollusc *Telescopium telescopium, Indian Journal of Physiology and Pharmacology*, 2001; **45** (2): 249- 252.,
15. Schillinger U and Lucke F K, Antibacterial activity of *Lactobacillus sake* isolated from meat, *Journal of Environmental and Applied Microbiology*, 1989; **55**:1901–1906.
16. Arputha Bibiana M, Selvamani P and Latha S, Identification and Appraisal of crude protein extracts from south Indian Marine Edible Bivalves for their potential bactericidal Property, *Asian Journal of Pharmaceutical and Clinical Research* , 2014; **7**(1): 233-236.
17. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 1970; **227**: 680-685.
18. Lowry O and Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent, *Journal of Biological Chemistry*, 1951; **193**:265-275.
19. Boobathy S, Ajithkumar TT and Kathiresan, Isolation of Symbiotic bacteria and bioactive proteins from the marine sponge *Callyspongia diffusa, Indian Journal of Biotechnology*, 2009; 8: 272-275.
20. Bartlett TC, Cuthbertson BJ, Shepard EF, Chapman RW and Gross PS, Crustins, homologues of an 11.5-kDa antibacterial peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*, *Marine Biotechnology*, 2002; **4**:278-293.
21. Renwrantz L and Stahmerge A, Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of heamocytes from *Mytilus edulis*, *Journal of Comparative Physiology*, 1983; **149**: 535-546.
22. Sumita S, Chatterji A and Das P, Effect of different extraction procedures on antimicrobial activity of marine bivalves: A comparison, *Pertanika Journal of Tropical Agricultural Science*, 2009; 32: 77-83.
23. Annamalai N, Anburaj RJ and Thavasi R, Antibacterial activities of green mussel (*Perna viridis*) and edible oyster (*Crassostrea madrasensis*), Research in Microbiology, 2007; **2**(12): 978-982.
24. Scotti PD, Dearing SC, Greenwood DR and Newcomb RD, Pernin: A novel, self-aggregating haemolymph protein from the New Zealand green-lipped mussel, *Perna canaliculus* (Bivalvia: Mytilidae), Comparative Biochemistry Physiology Part B: *Biochemistry and Molecular Biology*, 2001; **128**: 767-779.
25. Chandren BG, Ramesh kumar G and Ravichandren S, Antimicrobial activity from the gill extraction of pernaviridis(Linnaeues, 1758), *Global Journal of Biotechnology and Biochemistry*, 2009; **4**: 88-92.
26. Gopalakrishnan S, Vijayavel K, Nutritional composition of three estuarine bivalve mussels, *Perna viridis, Donax cuneatus and Meretrix meretrix*, *International Journal of Food Sciences Nutrition*, 2009; **60**(6):458-4.3.
27. Seo JK, Crawford JM, Stone KL and Noga EJ, Purification of a novel arthropod defencin from the American oyster *Crassostrea virginica*, *Biochemical and Biophysical Research Communication*, 2005; **338**: 1998-2004.