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APPRAISAL OF FIVE DIFFERENT PHENOTYPIC EDIBLE BIVALVES CRUDE PROTEIN EXTRACT FOR MICROBICIDAL POTENCY

M. ARPUTHA BIBIANA, K. GAYATHRI, P. SELVAMANI, S. LATHA
Department of Pharmaceutical Technology, Anna University Chennai, Regional Centre, BIT Campus, Tiruchirappalli.

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Abstract: The stretches of microbial infections are abiding from ancient days to till date with different symptoms and severity. Most of the modern medicines were failed to treat the infection without side effects. The resistance developed by the microbial strains is also harmfully increased. To overcome all these effects the research was focused upon bioactive natural products from various types of natural sources. Based on this idea we work towards identification of antimicrobial proteins from an edible bivalve (*Donax cuneatus*). Fresh bivalves of five different phenotypic characters were collected from the coast of Cuddalore. The bivalves were named as P1, P2, P3, P4 and P5. The fleshes of the bivalves were then removed and the crude protein was extracted from each type with 5% of cold acetic acid in water solvent, centrifuged at 4˚C and partially purified by ammonium sulfate precipitation. The precipitate was stored at -20˚C still evaluation. The precipitate was diluted in double distilled water and antimicrobial activity was assayed by well diffusion method against few strains of bacterial clinical isolates. All the extracts were potential against the tested microbial strains. The maximum zone of 26mm, 25mm and 20mm was observed with P5 extract against *Bacillus sp, Staphylococcus sp and Proteus sp respectively* and a minimum of 8mm zone was observed with P2 extract against *Pseudomonas sp, Salmonella sp, Shigella sp.* 1mg of protein in 1ml of P5 precipitate was estimated by Lowry's method. All the other types had the protein concentration not less than 0.9mg/ml. The SDS and FTIR studies also portrayed the presence of proteins in the crude extract of P5.

Keywords: Antimicrobial assay, Antimicrobial peptides, marine invertebrates, protein characterization.

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Corresponding Author: MS. M. ARPUTHA BIBIANA

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INTRODUCTION

Molluscs are far and wide dispersed all over the world namely slugs, whelks, clams, mussels, oysters, scallops, suits and octopus. Ocean includes a huge biodiversity of plants and animals which is predictable to be over 5,00,000 species more than twice over of the land species [1]. Molluscs are highly delicious seafood and they are also very good source for biomedically imported products.

This rich diversity of marine organisms assumes a great opportunity for the discovery of new bioactive substances. Thus the marine environment is an incomparable pool for bioactive natural products[2].

From 1960's approximately 300 bioactive marine natural products were filed for patent. Nearly 6,500 bioactive compounds have been isolated from the marine organisms[3].

Many of the bioactive compounds are found to be exhibiting antitumorous, antileukemia, antibacterial, and antiviral activities and are reported worldwide.

Though many bioactive natural compounds have been derived from invertebrates for various disorders and diseases the focusing of research now relies upon Anti-Microbial Peptides (AMPs). This is because of the overall inhibitory effect of them and the humoral natural defense of invertebrates against infections. They are also termed as "natural antibiotics". Generally they work with the innate immune mechanism of their own against many of the pathogenic microbes[4].

The screening of marine organism, especially marine bivalves for therapeutic drugs are of greater interest now a day. Hence, a broad based screening of marine bivalves for bioactive compounds is necessary. The present study was a preliminary step for the identification of novel antimicrobial peptides from the collected marine samples.

MATERIALS AND METHODS

Collection of bivalves

Five different types of marine edible bivalves (Fig: 1) were collected from the coast of cuddalore, TamilNadu and they were authenticated by Dr.A.Shanmugam, Professor, CAS in Marine Biology, Annamalai University, Parangipettai. The samples were identified as different phenotypes of *Donax cuneatus*. The samples were named as P1, P2, P3, P4 and P5.

Fig:1 Different phenotypes of *Donax cuneatus*

P1 P2 P3 P4 P5

Preparation of crude protein extract

The collected bivalves are brought to the laboratory and shells were broken and soft body was removed. The collected sample was extracted using 5% of cold acetic acid by manual homogenization [5]. Transfer the homogenate to centrifuge tubes and centrifuge at 7500rpm for 30minutes at 4°c. Then remove supernatant and store at -20ºC.

Partial purification of protein

Measure the volume of the supernatant obtained from centrifugation. Calculate the required amount of ammonium sulphate salt needed to saturate the solution at 100%. 60gm ammonium sulphate salt is needed for 100ml of saturated solution. Add the required salt to the solution slowly in small quantities and mix well continuously in ice bath supported magnetic stirrer after each addition. After addition of salts, centrifuge at 7500 rpm for 45 min. Discard the supernatant and dissolve the precipitate in few ml of double distilled water.

The dissolved precipitate was dialysed extensively in the distilled water and stored at -20ºC for further evaluation of the extract.

Antimicrobial Assay

Antimicrobial activity was determined by agar well diffusion method [6]. Nutrient agar plates were swabbed with the respective 24hrs broth culture of the organisms and kept for 15 minutes in laminar chamber for absorption of cultures. Wells were made in agar plates using a sterile cork bore of 5mm and 200µl of crude protein extract was added to each well. The plates were incubated at 37^0c for 24 hours and the diameters of the inhibition zone were measured in millimeter.

Minimal inhibitoryconcentration (MIC)

The broth tube dilution test is the standard method for determining levels of resistance to drugs like antibiotics. Serial dilutions of the crude extracts are made in a liquid medium which is

inoculated with a standardized number of organisms and incubated for a prescribed time. The lowest concentration (highest dilution) of crude extract preventing appearance of turbidity is considered to be the minimal inhibitory concentration (MIC). At this dilution the antibiotic is bacteriostatic.

The MIC tubes were further carried out for Minimal Bactericidal concentration evaluation.

Minimal bactericidal concentration (MBC)

From each MIC broth tube without visible growth, a aliquot a loop of broth was streak plated on a nutrient agar plate. Record the dilution of the streak plated MIC tube on agar plates and incubate at 35^0C for 24hrs. Following overnight incubation examine the MBC plate for colony growth or lack of growth for each dilution subculture. No growth indicates that the antibiotic was bactericidal at that dilution. Growth indicates that the antibiotic was bacteriostatic but not bactericidal at that dilution.

SDS PAGE Analysis

The molecular weight of the proteins in crude extracts were confirmed by the SDS PAGE analysis [7] with the molecular marker ranging from 3.5 to 250 kda.

Estimation of protein concentration

The concentration of protein in the crude sample is estimated by the Lowry's [8] method using BSA as standard.

FTIR

The crude protein extract was subjected to FTIR characterisation under suitable conditions. The Functional groups of proteins were identified in a different wavelength.

RESULTS AND DISCUSSION

Antimicrobial Susceptibility testing

All the five crude 5% acetic acid extracts of *Donax cuneatus* were evaluated for its antimicrobial activity against pathogenic microorganisms. The results of all the extracts can be interpreted from the Table: I.

Table: I Zone of inhibition of crude Protein Extracts

S.NO	MICRO ORGANISM	ZONE OF INHIBITION (mm)				
		P1	P2	P3	P4	P5
1	*Penicillium sp*	10	9	13	12	17
2	*Aspergillus sp*	10	9	16	16	16
3	*Shigella sp*	10	8	15	14	17
4	*Salmonalla sp*	9	8	15	10	17
5	*Bacillus sp*	11	9	14	23	26
6	*E.coli*	11	9	16	15	18
7	*Pseudomonas sp*	10	8	12	11	17
8	*Staphylococcus sp*	11	13	10	22	25
9	*Klebsilla sp*	12	11	10	13	17
10	*Streptococcus sp*	12	11	8	15	18
11	*Proteus sp*	11	12	10	11	20

All the tested pathogenic microbial cultures were susceptible to the five crude protein extracts of *Donax cuneatus* . The maximum inhibitory effect of 26 mm was observed with the P5 extract against *Bacillus sp (*Fig: 2a). *Staphylococcus sp* showed good susceptibility of 25mm with the P5 extract (Fig: 2b). The highly pathogenic *Streptococcus sp* and *Proteus sp* (Fig: 2e) showed 18mm and 20mm zone of inhibition respectively against P5 extracts. The Gram negative *Shigella sp, Pseudomonas sp* and *Salmonella sp* were also inhibited with 17mm of zone of inhibition (Fig: 2b, 2c). The fungal strains *Asperillus sp* and *Penicillium sp* were also equally inhibited with 16mm and 17mm zone of susceptibility (Fig: 2d). The minimum zone of inhibition was observed with the *Bacillus sp* of 7mm. Some other cultures like *Streptococcus sp , Shigella sp* and *Salmonella sp* showed minimum inhibitory effect against P2 and P3 extract. The maximum activity was shown by the P5 extract of the *Donax cuneatus* and all the further analysis was carried out with the same.

The extracts of mollusc *Mytilus galloprovincialis inhibits the growth of bacteria by fungi by* the phagocytosis activity which is found similar to mammalian phagocytosis activity against insects[9]. The Cg pep 33 antimicrobial peptide exhibits activity against Gram positive, Gram negative bacteria and fungi which is isolated from the digestion part of mollusks[10]. Many of the mollusks from various parts of the world were identified for their potential antimicrobial property to overcome the problem of resistance. The organic and aqueous extracts of nearly 58 samples of molluscs were found to have beneficial amount of microbicidal activity against many bacteria and fungi [11,12]. Effective chemotherapeutic agents were also developed from the antimicrobial peptides identified from marine invertebrates[13].

Fig: 2 Plates Showing Antimicrobial Effect

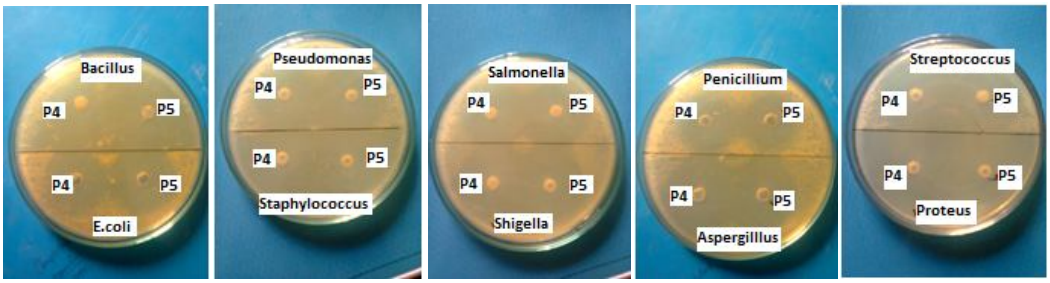


Fig: 2a **Fig:2b** **Fig: 2c** **Fig: 2d** **Fig: 2e**

Minimal Inhibitory Concentration

The minimal inhibitory concentration of the P5 crude protein extract was evaluated at various concentrations against few of the bacterial cultures that showed maximum zone of inhibition in well diffusion method. The values can be interpreted from Table: III

Table: III Minimal Inhibitory Concentration of P5 crude extract

S.NO	ORGANISM	MIC (ml)
1	*Proteus sp*	0.2
2	*Klebsilla sp*	0.1
3	*Salmonalla sp*	0.1
4	*Shigella sp*	0.1
5	*E.coli sp*	0.1
6	*Bacillus sp*	0.1
7	*Pseudomonas sp*	0.4
8	*Staphylococcus sp*	0.3

The *Pseudomonas sp* was inhibited at 400µl of the crude extract and *Staphylococcus sp* at 300µl. The growth of *Bacillus sp, E.coli sp, Shigella sp, Salmonella sp* and *Klebsiella sp* were inhibited at the lowest concentration of 100µl of extract and the microbial strain *Proteus sp* was inhibited at 200µl of the extract. The minimal inhibitory concentrations of the tested microbial strains were proven that the crude extract may be developed as a promising protein antibiotic with good potency.

Minimal Bactericidal Concentration

The MIC was followed with the MBC of the crude extracts to determine their bactericidal effect upon the microbial cultures. The results of MBC can be interpreted from the Table:IV

Table:IV Minimal Bactericidal Concentration of the P5 crude extract

S.NO	ORGANISM	MBC
1	*Proteus sp*	0.2
2	*Klebsilla sp*	0.1
3	*Bacillus sp*	0.1
4	*Salmonalla sp*	-
5	*Shigella sp*	-
6	*E.coli*	-

The microbial strains *Salmonella sp, Shigella sp and E,coli* didn't show any bactericidal effect upon the activity of the crude extracts. The MIC of *Proteus sp* coincides with the MBC result. The MBC of *Klebsiella sp and Bacillus sp* remains at 100µl alike MIC.

Protein Estimation

The optical density of P1, P2, P3, P4 and P5 observed at 640nm were 0.99, 0.85, 1.05, 0.85 and 1.23 respectively. The maximum concentration of 1.mg/ml 1.23of protein was observed with P5 crude extract. All the other extracts have protein concentration of not less than 0.85mg/ml. (Table:II)

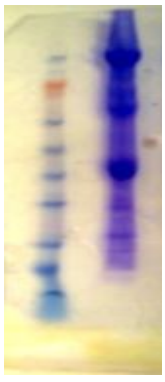
Table:II Concentration of Protein in crude extracts

Protein extract	Optical density (640nm)	Concentration (mg/ml)
P1	1.032	0.99
P2	1.099	0.85
P3	1.035	1.05
P4	1.028	0.85
P5	1.121	1.23

SDS-PAGE analysis

The P5 crude extract of edible bivalve was subjected to molecular weight determination by SDS - PAGE analysis. Six bands were observed as a result of the analysis. The bands were separated at range of molecular weight 3, 6, 18, 17, 38, 89, and 62 kda (Fig:3). These were compared with the protein marker and the separated proteins were identified to resemble the molecular weight of Lysozyme, Myoglobin red, Carbonic anhydrase, Alcohol dehydrogenase, BSA and Myosin respectively.

Fig:3 SDS - PAGE Analysis



3 k Da
4 k Da
17 k Da
28 k Da
38 k Da
49 k Da
62 k Da
98 k Da
188 k Da

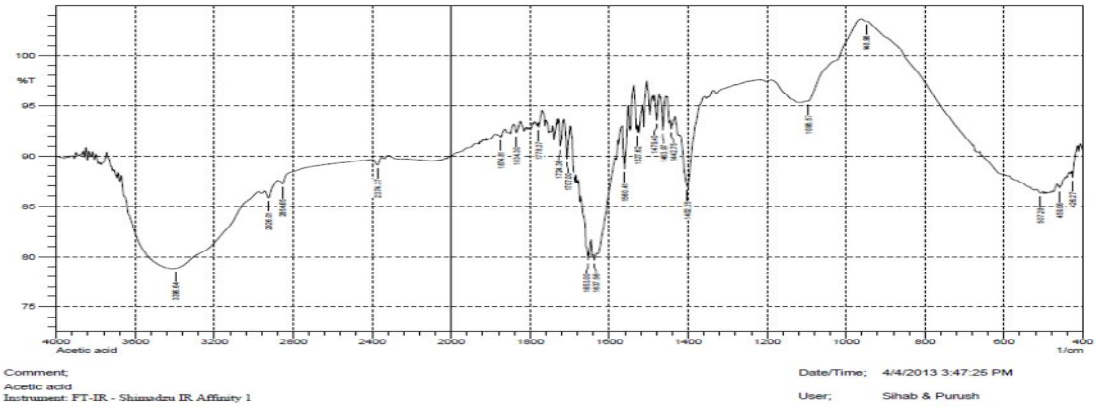
FTIR characterisation

FTIR characterization of crude protein showed the presence of functional groups of protein (Fig: 4) like NH, COOH, -CO-, CH, SH, C=O, C=C and other R groups at appropriate wave length and stretching. The results can be inferred from the Table: V.

Table: V IR analysis of crude protein extract

S.No	Functional Group	Absorption Spectrum
1	Amino (NH)	3396
2	Alkyl (CH)	2926
3	Alkyl (CH)	2854
4	(SH)	2374
5	Carboxylic acid (COOH)	1778
6	Ketone (-CO-)	1724
7	Aldehyde (C=O)	1707
8	Amine	1560
9	Aromatic C=C	1527
10	Cyclic ether	1095

Fig: 4 FTIR report of Crude Protein Extract



CONCLUSION

All the crude protein extracts showed considerable role of microbicidal property. The variations in the inhibitory effect of the extracts may be due to the change in their phenotypes of the species. Though they are all collected at the same tidal area their polymorphism might play researchable role in the antimicrobial property.

Equally all the Gram positive and Gram negative bacteria showed good inhibitory effect against the tested protein extracts. The proteins and peptides act upon the cell wall of both the microbial pathogens and hence prevent it from further multiplication. Apart from inhibiting bacterial cell wall it also inhibits fungal culture by the same mechanism of action upon the fungal cell wall. In future considering this work it can be developed into a better biopharmaceuticals for infections.

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