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Research Article

IDENTIFICATION AND APPRAISAL OF CRUDE PROTEIN EXTRACTS FROM SOUTH INDIAN MARINE EDIBLE BIVALVES FOR THEIR POTENTIAL BACTERICIDAL PROPERTY

M.ARPUTHA BIBIANA*, P.SELVAMANI AND S.LATHA

Department of Pharmaceutical Technology, Anna University, BIT Campus, Tiruchirappalli, Tamilnadu, India.
Email: arputha_bibiana@yahoo.com

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ABSTRACT

Objectives: The crude protein extracts of edible marine invertebrates *Pitar erycina* and *Donax cuneatus* collected from the coastal area of South India were evaluated for its antimicrobial potency against broad spectrum of bacterial pathogens.
Methods: The crude proteins were extracted from the flesh with 5% cold acetic acid and buffers at acidic, neutral and basic pH. The crude was partially purified by ammonium sulfate precipitation method and the precipitate was stored at -20˚C still evaluation. The antimicrobial property was assessed by well diffusion method. Both the edible oyster extracts showed inhibitory effects against the tested bacterial and fungal strains.
Results: The maximum zone of 24mm was observed with *Donax cuneatus* acidic extract against *Bacillus subtilis* and a minimum of 17mm zone was observed against *Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri.* The *Pitar erycina* acidic extract showed the maximum activity against *E.coli* and *Shigella flexneri* with 17mm and 15mm zone respectively. The antimicrobial potency of buffer extracts of the two bivalves was comparingly low with that of the acidic extracts. 1.23mg/ml protein was estimated in *Donax cuneatus* acidic extract.
Conclusion: The crude extracts of protein from edible bivalves showed potent bactericidal property against highly pathogenic species, which may helps to find a safe novel protein/peptide antibiotic.

Keywords: Edible bivalve, Marine invertebrate, Antimicrobial Assay, Antimicrobial Peptides.

INTRODUCTION

Mollusks are widely distributed throughout the world and many representative in the marine and estuarine ecosystem namely slugs, whelks, clams, mussels, oysters, scallops, suits and octopus. Nearly all of the mollusks are consumed by the coastal area people in their normal diet [1]. Unknowingly they have rich medicinal value that includes antitumorous, antileukemia, antibacterial, and antiviral [2].

Though many bioactive natural compounds have been derived from invertebrates for various disorders and diseases the focusing of research now relies upon Antimicrobial proteins. 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 [3].

The amino acid composition of peptides have amphipathicity, cationic charge and size allow them to attach to and insert into membrane bilayers to form pores by 'barrel-stave', 'carpet' or 'toroidal-pore' mechanisms. In fact several observations suggest that translocated peptides can alter cytoplasmic membrane septum formation, inhibit cell-wall synthesis, inhibit nucleic-acid synthesis, inhibit protein synthesis or inhibit enzymatic activity. Consisting no more than a dozen aminoacids, rapidly produced and diffusible they seem ideal for fast and efficient defense against microbes.

The present study was subjected to identification of bactericidal proteins from the edible marine invertebrates of South Indian coastal area especially Tiruchendur and Kanyakumari of Tamilnadu. The edible varieties of bivalves preferably unexplored were considered for the therapeutic protein identification.

Materials and Methods

Collection of Marine Edible Bivalves

Edible varieties of bivalves were collected from the coast of TamilNadu, India. Two varieties of bivalve namely *Donax cuneatus* and *Pitar erycina* were collected and authenticated from CAS for Marine biology, Annamalai University, Parangipettai, TamilNadu.

Extraction of Microbicidal peptides

Fresh samples were transferred to the laboratory immediately maintaining the samples at 8˚C. The samples were washed with distilled water and the flesh of all the samples were taken by breaking the shells of the bivalves. Microbicidal peptides were prepared from the whole body tissue of bivalves using 5% acetic acid in water and buffers (Tris Hcl, Tris base and Phosphate buffer) at pH 4, 7 and 9 by simple homogenization following the procedure of Carlos *et al* with slight modifications in percentage of acetic acid and choice of buffers. The homogenized mixtures were centrifuged at 4˚C in 7500 rpm for 30 min. The supernatant were partially purified for peptides by ammonium sulfate precipitation method at 85% saturation and again centrifuged at 4˚C in 7500 rpm for 45 min. The precipitates were dialysed extensively against double distilled water and the retentate were stored at -20˚C still further evaluation.

Antimicrobial Assay

The antimicrobial potency of the crude extracts was evaluated by well diffusion assay. 100µl of each extract was evaluated for its antimicrobial potency against the microbial pathogens. The zone of inhibition was measured in millimeter. The assay was repeated in triplicate and the averages of the three were given as results.

MIC and MBC determination

The minimal inhibitory concentration was determined by broth tube dilution assay using standard protocols. Crude extracts at various concentrations from 0.1ml to 0.5ml were evaluated for inhibitory level against bacterial pathogens.

The MIC tubes were further carried out for Minimal Bactericidal concentration evaluation. Loops of cultures from the MIC tubes were transferred to the nutrient agar plates and the growth was monitored after 24hrs of incubation.

SDS PAGE Analysis

The proteins and peptides in the crude extract were confirmed by SDS PAGE analysis with the molecular marker ranging from 3.5 to 200 kda.

Estimation of protein concentration

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

Results

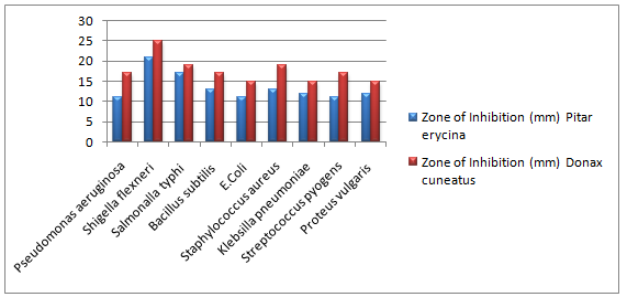
Antimicrobial Assay

Effect of Acetic acid Extracts

The crude extracts of the two species showed promising antimicrobial susceptibility to all the tested strains at the concentration of 100µl per well. The acetic acid extracts were measured with higher zone of inhibition comparing with the buffer extracts.

All the tested pathogenic microbial cultures were susceptible to the crude protein extracts of *Donax cuneatus (DC)* and *Pitar erycina (PE)*. The maximum inhibitory effect of 25mm was observed with the *Donax cuneatus* extract against *Shigella flexneri (fig: 2C)* and 19mm against *Salmonella typhi and Staphylococcus aureus (fig: 2A)*. The *Pseudomonas aeruginosa* and *Streptococcus pyogens* is the next pathogen inhibited with 17mm of zone of inhibition by the extract of DC. The other tested strains were inhibited with not less than 15mm of inhibitory effect by DC extract and 11mm by PE extract.

Fig. 1: Graphical representation of the bactericidal activity of crude acetic acid protein extract of DC and PE



Plates showing maximum activity against acidic extracts

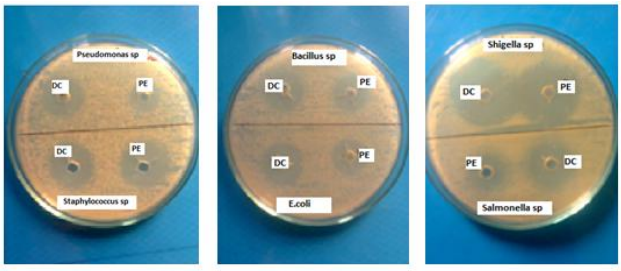


Fig: 2A Fig: 2B Fig: 2C

The extracts of PE also showed antimicrobial susceptibility against all the pathogenic strains but the activity was comparingly low with that of DC. The maximum inhibitory effect shown by PE extract was 21mm against *Shigella flexneri* and 17mm against *Salmonella typhi.* The minimum of 11mm zone was observed with the dreadly pathogenic *Pseudomonas sp, E.coli and Streptococcus sp.*

Equally all the Gram positive and Gram negative bacteria showed good inhibitory effect against the tested crude protein extracts. In overall concern the effect of antimicrobial property of the DC extracts were very active against the pathogenic strains than PE extracts.

Effect of Tris Hcl Extracts

The Tris Hcl buffers were prepared at their acidic, neutral and basic pH and the inhibitory levels were measured against the pathogenic strains (Table 1).

Table 1: Antimicrobial Effect of Tris Hcl buffer Extract

S.No	Microbial Pathogens	Zone of Inhibition (mm) of Tris Hcl Buffer Extract					
		Pitar erycina			*Donax cuneatus*		
		pH 4	pH 7	pH 9	pH 4	pH 7	pH 9
1	*Salmonella typhi*	8	9	8	8	7	9
2	*Bacillus subtilis*	9	5	9	9	6	7
3	*E.coli*	7	6	9	9	6	8
8	*Pseudomonas aeruginosa*	8	5	5	8	7	6
5	*Staphylococcus aureus*	10	9	8	12	5	7
6	*Streptococcus pyogens*	8	5	9	3	8	3
7	*Proteus vulgaris*	7	6	7	8	5	7
8	*Klebsiella pnemoniae*	6	7	6	8	7	8
9	*Shigella flexneri*	6	5	7	6	7	7

The maximum susceptibility was observed with the *Staphylococcus sp* of around 10mm of PE extract at the acidic pH. The DC extract showed 12mm zone of inhibition against *Staphylococcus sp* at acidic pH range whereas the other extracts showed maximum activity against pathogens only in acidic and alkaline pH.

Effect of Tris Buffer Extracts

The antimicrobial effect of Tris base buffer was also measured at various buffer ranges (Table 2) and the results were tabulated.

Table2: Antimicrobial Susceptibility of Tris Buffer Extracts

S.No	Microbial Pathogens	Zone of Inhibition (mm) of Tris Buffer Extract					
		Pitar erycina			*Donax cuneatus*		
		pH4	pH7	pH9	pH4	pH7	pH9
1	*Salmonella typhi*	8	7	9	8	7	7
2	*Bacillus subtilis*	8	7	8	7	7	8
3	*E.coli*	8	7	9	8	8	9
8	*Pseudomonas aeruginosa*	4	6	7	7	6	8
5	*Staphylococcus aureus*	9	7	8	9	6	8
6	*Streptococcus pyogens*	7	5	3	8	7	7
7	*Proteus vulgaris*	7	5	9	9	8	7
8	*Klebsiella pneumoniae*	8	7	8	6	7	8
9	*Shigella flexneri*	7	6	7	7	6	5

The maximum activity was noted with the *Staphylococcus sp* of 9mm for acidic pH and 9mm for *Salmonella typhi, E.coli and Proteus vulgaris.* The extracts of *Donax cuneatus* showed maximum activity to *Proteus sp* and *Staphylococcus sp* in acidic pH and *E.coli* in alkaline pH.

Effect of Phosphate Buffer

The phosphate buffers were prepared at various ranges of pH and the susceptibility was promisingly measured with good results and the results were tabulated in the Table 3.

Table 3: Antimicrobial Susceptibility of Phosphate Buffer

S.No	Microbial Pathogens	Zone of Inhibition (mm) of Phosphate Buffer Extract					
		Pitar erycina			*Donax cuneatus*		
		pH4	pH7	pH9	pH4	pH7	pH9
1	*Salmonella typhi*	8	8	8	7	6	8
2	*Bacillus subtilis*	9	7	9	9	7	9
3	*E.coli*	9	8	8	8	7	6
8	*Pseudomonas aeruginosa*	8	7	7	6	7	8
5	*Staphylococcus aureus*	10	6	8	7	5	8
6	*Streptococcus pyogens*	6	6	7	6	6	7
7	*Proteus vulgaris*	7	5	9	9	8	7
8	*Klebsiella pneumoniae*	8	7	8	6	7	8
9	*Shigella flexneri*	7	6	7	7	6	5

The Gram positive pathogenic *Staphylococcus aureus* was inhibited with 10mm zone of inhibition by the acidic pH extracts of PE. The extracts of *Pitar erycina and Donax cuneatus* showed higher activity to *Bacillus subtilis* in acidic and alkaline pH. The *Staphylococcus sp* was also susceptible with the inhibitory effect of 14mm.

The effect of the antibacterial property of the buffer extracts were comparatively depicted in Fig: 3. The Tris Hcl buffer extracts showed potent activity at its acidic pH of DC and acidic pH of PE also showed good activity against *Staphylococus aureus*. In overall activity chart the acidic extracts of both the species were good in their activity compared to the other extracts. They were active against the pathogenic *Bacillus sp, E.coli and Staphylococcus sp.*

MIC and MBC evaluation

The extracts of both the species was evaluated for its MIC and MBC activity. The extracts inhibits the bacterial strains with the minimum inhibitory concentration of not less than 100 µl of the extract. The *Pseudomonas sp* and the *Staphylococcus sp* were inhibited at 200 µl and 300 µl of *Pitar erycina* extract and 400 µl, 300 µl respectively for the *Donax cuneatus* extract. Unfortunately the extracts just inhibit the growth of pathogens at higher concentrations and they were not killed. No potent MBC were found for the *Staphylococcus sp* and the *Pseudomonas sp*. The inhibitory and bactericidal concentration remains same for both the extracts of PE and DC against *Proteus sp, Klebsiella sp and Salmonella sp* (Table:4).

Table 4: MIC and MBC of crude protein extracts of *Pitar erycina* and *Donax cuneatus*

S.No	Pathogens	*Pitar erycina*		*Donax cuneatus*	
		MIC	MBC	MIC	MBC
1	*Proteus vulgaris*	0.2	0.2	0.2	0.2
2	*Klebsilla pneumonia*	0.1	0.1	0.1	0.1
3	*Salmonalla typhi*	0.1	0.1	0.1	0.1
4	*Shigella flexneri*	0.1	-	0.1	-
5	*Streptococcus pyogens*	0.1	-	0.1	-
6	*E.coli*	0.2	-	0.1	-
7	*Bacillus subtilis*	0.1	-	0.1	-
8	*Pseudomonas aeruginosa*	0.2	-	0.4	-
9	*Staphylococcus aureus*	0.3	-	0.3	-

SDS PAGE Analysis

The SDS analysis with the marker range 3.5 Kda to 200 Kda revealed the results with the separation of protein at 3.5, 14, 17, 34, 49, and 62 kda. The protein bands of both the species, *Donax cuneatus* in Lane 2 and 4 and *Pitar erycina* in Lane 3 and 5 coincide with each other with the molecular marker in Lane 1 (Fig:3). The concentration of protein in the acetic acid extracts were higher in the gel comparing with the buffer extracts.

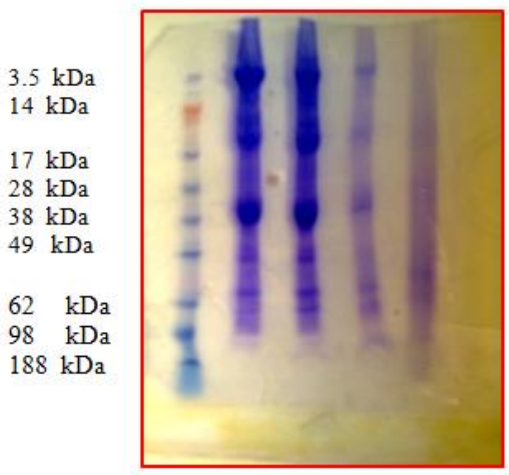


Fig3: SDS PAGE for the crude extracts

Protein Quantification

The quantity of protein in the extract was determined with the help of Lowry's method measured the concentration of proteins as 0.9mg for *Pitar erycina* and 1.23mg for *Donax cuneatus.*

DISCUSSION

The crude solvent extracts and protein extract of many marine invertebrate like *Callyspongia diffusa*, *Sigmadocia fibulatus*, *Trochus tentorium, Scylla serata* showed potent antimicrobial activity against many of the tested bacterial strains and fungal strains [5,6] [7] and [8].

The bacterial cell wall is made up of phospholipids like phosphatidylglycerol and cardiolipin. These phospholipids are heavily negatively charged and hence they are attracted towards the positively charged antimicrobial proteins. The interactions between the positive charges of antimicrobial proteins and the negatively charged phospholipids of bacterial cell wall are mainly due to electrostatic interactions [9]. Apart from charges the hydrophobic nature of the antimicrobial proteins also plays a minor role in the destruction mechanism [10]. Hence by the action of proteins and peptides act upon the cell wall of the microbial pathogens it significantly prevents the pathogens from further multiplication.

Although antimicrobial peptides from molluscs were extensively studied, most of the research has focused on the bivalves such as *Mytilus edulis* and *Mytilus galloprovincialis.* These species are capable of synthesizing defensis, mytilins, and the antifungal peptide mytimycin, all of which having a molecular weight below 10 kDa [11]. Only one antimicrobial peptide from mollusks with molecular mass approximately 10kDa has been reported. This is the case of the big defensin identified from the bay scallop *Argopecten irradians* [12].

The presence of microbicidal peptides, their activity and concentration of proteins in the prepared extract were well cleared in the above work. The purification and characterization of the above said crude protein were in progress for future publications.

Conclusion

The bactericidal proteins identified from the edible marine invertebrates *Donax cuneatus and Pitar erycina* was found to have a very good antimicrobial potency against the major human pathogenic microorganisms. The identification of this protein therapeutics will help researchers in the development of novel antibiotics. The protein molecule with its own property may help in

killing the pathogenic microbes and preventing the pathogens from developing into resistant strains.

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