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Research Article

IDENTIFICATION AND ISOLATION OF GLYCINE, ALANINE AND ASPARAGINE RICH SECRETORY PROTEIN WITH ANTIBACTERIAL EFFICIENCY FROM *DONAX CUNEATUS*

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ABSTRACT

The crude protein was extracted from marine edible bivalve *Donax cuneatus,* precipitated and further dialysed against PBS in dialysis membrane having MW cut off of 3 KDa. The extract was evaluated for its antimicrobial activity against highly pathogenic Gram positive and Gram negative bacteria. It was found to be active against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The purified fractions obtained from anion-exchange column were evaluated for their activity against the aforesaid bacteria. The highly active fraction against *Staphylococus aureus* with the zone of inhibition of 12 mm was identified and further purified and characterised. The active fraction was enriched with glycine, alanine and asparagine aminoacids.

Keywords: *Donax cuneatus*, antimicrobial peptides, ion-exchange chromatography, MALDI TOF, sequence analysis.

INTRODUCTION

The promising antimicrobial compounds are antimicrobial peptides (AMPs) which are small sequences comprising amino acids ranging from 10-100 residues that eminently kill the pathogens and could be permanent solution for infections of bacterial origin especially. Though the pathogens develop resistance against each of the existing antibiotics, the specific bactericidal properties of the AMPs will elude the resistance property and end up infection without any side-effects [(1)].

Novel peptides from various marine sources like sponges, ascidians, sea anemones, seaweeds, *etc*., are isolated and they are submitted to clinical trials. The majority of antimicrobial peptides are being cationic species; few Anionic Anti Microbial Peptides (AAMPs) are predicted that originated as a result of eukaryotic innate immune response [(2)].

Though invertebrates are deficient of the acquired immune system, they solely depend on their innate immune system to counteract invading infectious pathogens. The invertebrates prevent themselves from both endogenous and exogenous bacteria with the help of innate immunity[(3)]. Keeping in mind the amazing evolutionary achievement of this group of organisms, it is evident that invertebrate innate immune mechanisms are extremely effective. The physical barriers, effectors molecules and immune cells like neutrophils and macrophages are grouped under the innate immune system. Most of the anionic peptides derived are of epidermal origin of the eukaryotes[(4)]. The AAMPs work with the diverse range of attacking mechanisms against microorganisms, which includes translocation across the cell membrane to enroll with the intracellular sites of antimicrobial action. In most of the cases, the microbial membrane itself is the major site of action for AAMPs, which induce the rupturing of membranes by carpet-type mechanisms before acting against intracellular sites[(5,2)]. Apart from antibiotic property the peptides are also used in the commercial applications of biocides.

Many of the mollusks from various parts of the world were identified for their potential antimicrobial property to overcome the problem of resistance. The action of peptides might be by the process of phagocytosis which is found to be similar with mammalian phagocytosis activity against insects[(6)]. The antimicrobial peptide Cg pep 33 isolated from the digestion part of mollusks was also reported to possess antimicrobial property against Gram positive, Gram negative bacteria and fungi[(7)]. Even the aqueous extracts of nearly 58 samples of molluscs were found to have beneficial amount of microbicidal activity against many bacteria and fungi. Molluscs were found to be such a rich source of protein[(8,9)] and many of the effective chemotherapeutic agents were also developed and are in trials from the marine invertebrates[(10)]

The present study is aimed at to isolate and characterize naturally occurring bioactive proteins or peptides of innate immune origin from the marine invertebrate *Donax cuneatus* with its potential antimicrobial property.

MATERIALS AND METHODS

Collection of samples

The marine edible bivalves *Donax cuneatus* were collected from the shores of Tiruchendur, Tamil Nadu. The specimen was authenticated from Centre for Advanced Studies in Marine Biology CAS, Annamalai University, Parangipettai, Tamil Nadu, India. The collected bivalves were carefully transported to laboratory in freezer box.

Extraction of crude proteins

The shells were broken and tissues were collected, washed with distilled water and chopped into small pieces. They were homogenized with prechilled mortar and pestle for crude protein extract, with cold 5 % acetic acid in water as extraction solvent. The extract was centrifuged at 8000 rpm for 30 min at 4^{o}C. The protein in the supernatant was concentrated by ammonium sulphate (Merck, Mumbai, India) precipitation method. While

increasing the concentrations of salt the protein in the supernatant precipitates sharply. Hence the precipitation was carried out with 80% saturation in cold condition [11]. Then the mixture was centrifuged at 8000x*g* for 40 min at 4°C. The precipitated proteins were diluted with double distilled water and extensively dialyzed against distilled water using dialysis membranes of molecular cut off 3KDa (Sigma Aldrich, USA) The crude precipitate was lyophilized and stored for evaluation of antimicrobial activity against the clinical isolates.

Agar well diffusion assay

The well diffusion method was employed for determination of antibacterial effect of crude extracts against the bacterial strains namely *Proteus vulgaris, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri, Streptococcus pyogenes, E.coli, Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus aureus* obtained from Department of Microbiology, Bharathidasan University, Tiruchirappalli, TamilNadu. The nutrient agar plates were inoculated with the 24 hrs cultures grown in nutrient broth tubes. The agar plates were punched with a five millimeters diameter wells and the crude protein extracts of 100µL were filled in the wells. The concentration of the extracts was 100 µg/ml. The standard antibiotic chloramphenicol (Himedia, Mumbai) was used as the positive control at a concentration of 10 µg/ml in distilled water. The assay was repeated thrice and the results were recorded as zone of inhibition after incubation at 35 ± 2°C for 24 hrs. The values were calculated as mean ± SD with n=3 in GraphPad Prism version 6.0 30 days trial software.

Bioassay guided fractionation of proteins

The acidified-precipitated protein was purified by anion-exchange column chromatography. DEAE cellulose (Sigma Aldrich, USA) was equilibrated with 20 mmol L^{-1} sodium phosphate buffer (pH 7.0) and packed in a polypropylene column (2.5 × 9.5 cm). The lyophilized protein sample, 500 mg, was dissolved in the above buffer and centrifuged at 12,000 rpm for 15 min at 4°C. Then the protein sample (500 µL) was sterilized by filtration in 0.22 µm Millipore membranes (Sigma Aldrich, USA) and loaded on to the column. After washing the column with the same buffer, the bound proteins were eluted by a linear NaCl gradient (0–0.5 mol L^{-1}) in phosphate buffer with the flow rate of 1 mL. The presence of the bioactive pure protein fraction was monitored by assaying all fractions for antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The bioactive fractions with antimicrobial activity were pooled and concentrated by lyophilisation.

Reverse Phase – High Performance Liquid Chromatography (RP – HPLC)

The pure fraction separated by anionic column was further purified in a C_{18} column (Vydac) system with a linear acetonitrile gradient of 5 – 95% in 0.1% trifluoroacetic acid at a flow rate of 0.8 mL min^{-1} at a wavelength of 230 nm.

Minimal inhibitory concentration and minimal bactericidal concentration

The minimal inhibitory concentration (MIC) was determined by broth tube dilution assay using standard protocols [12] .Crude extracts at various concentrations from 10 µg/mL to 50 µg/mL in distilled water were evaluated for determination of inhibitory level against bacterial pathogens.

The MIC tubes were further tested for minimal bactericidal concentration (MBC) on the nutrient agar plates. A single loop of culture from the MIC tubes was transferred to the nutrient agar plates and the growth was monitored after 24 hrs at 37 ˚C of incubation.

Estimation of protein

The concentration of the protein in the crude and fraction was estimated by the standard protocol of Lowry *et al*. [13]

SDS-PAGE

The crude extract and active fractions were subjected to SDS/PAGE analysis using a vertical slab gel apparatus following the protocol of Laemlli *et al*. [14] with the stacking gel containing 3 % acrylamide and the resolving gel with 15 % acrylamide (0.75-mm thickness). A wide range marker (6.5 to 97.4 KDa, Genei, Bangalore) was used as a standard.

MALDI TOF MS

The MALDI-TOF mass spectra were used for the analysis of peptide mass fingerprinting and MS/MS ion search. The trypsin digested purified protein solution was used for the MALDI-TOF MS analysis, recorded on an AB SCIEX Voyager DE Pro MALDI-TOF (Applied Biosystems, USA) time-of-flight spectrometer, with a pulsed nitrogen laser (337 nm, 3-ns pulse width). The spectra were recorded in the linear, positive high-mass mode. The pure lyophilized peptide fraction of 1µL was diluted with 2 µL of alpha-cyano-4-hydroxycinnamic acid and formed as a peptide matrix and transferred to a stainless steel target and dried under gentle vaccum. The steel plate was then washed with 1 µl of 0.1 % trifluoroacetic acid [15].

Peptide mass fingerprinting and MS/MS ion search

The mass value obtained from MALDI TOF was subjected to identification of the peptide sequences in matrix science software. The selected peptide masses were submitted to Mascot search database [16] in matrix science and the sequence of the proteins were identified. The protein masses were matched against the SWISSPROT database using 100 ppm mass tolerance, limited to the metazoan proteins with a fixed modification of carbamidomethyl and oxidation as a variable modification.

Determination of Physicochemical property

The property of the identified sequence like charge, ratio of hydrophobicity, percentage of each aminoacid in the sequence was predicted with the help of the online free antimicrobial peptide database [17] of University of Nebraska Medical Center, Omaha, USA. The similar AMP sequences were also predicted from the same AMP database.

RESULTS

Antibacterial potentiality testing

The Gram positive bacteria *Staphylococcus aureus, Bacillus subtilis* and the Gram negative bacteria *Pseudomonas aeruginosa* were highly susceptible to the crude protein extracts of *Donax cuneatus*. The degree of inhibition of *P.aeruginosa* was comparingly low with the *S.aureus* and *B.subtilis.* The tested gram negative bacteria were inhibited with less zone of inhibition compared to the gram positive bacteria (Table:1).

MIC and MBC of crude proteins

The growth of *S.aureus* was inhibited at 30µg/mL and the growth of *B.subtilis* was inhibited at 10µg/mL. The growth of the bacteria alone was inhibited and it doesn't showed considerable *MBC* range. The *MIC* of gram negative bacteria *E.coli, Pseudomonas aeruginosa* and *Proteus vulgaris* was observed to be 20µg/mL. The pathogen *Proteus vulgaris* alone showed *MBC* at the same concentration of 20µg/mL whereas the other pathogens fail to show bactericidal activity.

Even though the other tested bacterial strains showed zone of inhibition lower than *S.aureus* and *B.subtilis* the growth of them were inhibited at a lower concentration of 10 µg/mL and the

MBC of *Proteus vulgaris, Klebsiella pneumoniae* and *Salmonella typhi* were also observed at 20 µg/mL, 10 µg/mL and 10 µg/mL respectively (Table 2).

Quantification of crude and isolated protein

The crude extracts of *Donax cuneatus* were estimated for its protein content by following standard Lowry's method and the concentration was calculated quantitatively using BSA (Bovine Serum Albumin) as standard. The concentration of the protein was found to be 1mg/mL of the crude extract.

All the obtained fractions were estimated for their protein concentration using microplate reader (Epoch, Biotek, USA) at 280nm. The six high protein concentrated fractions obtained through anion exchange chromatography were evaluated for the antimicrobial assay against the gram positive bacteria *Staphylococcus aureus*.

Identification of active fraction

The potency of pure fractions was evaluated with the help of *E. coli* in many of the previous reports. In our study the antimicrobial activity was studied with *S. aureus* and *P. aeruginosa*. The 3rd fraction with high protein concentration separated in DEAE cellulose column (Figure 1) was identified to be highly susceptible against the tested pathogens (Figure 2).

The pure fraction further separated by RP-HPLC showed two peaks (Figure 3) which was separated and evaluated for its antibacterial property. The two fractions showed minimum activity compared to the activity of fraction in combined form (Figure 4).

Hence the 3rd fraction obtained as a result of anion exchange column was processed for further characterisation.

SDS PAGE analysis

The SDS-PAGE analysis clearly reveals the presence of low molecular mass peptides in the pure 3rd fraction as a single band with molecular mass near 30 KDa (Figure 5).

The single band in lane B identifies the pure protein fraction isolated as a result of anion exchange chromatography.

MALDI TOF analysis

The trypsin digested anionic column separated pure fraction subjected to MALDI-TOF MS was identified with multiple ions. The molecular mass of the fraction from the spectra was identified as 10.5 KDa. The obtained mass values were subjected to mascot search for peptide mass finger printing. The molecular mass showed highest match score with the membrane secretory protein of marine species *Haliotis asinina* as a result of mascot search. As the protein was rich in glycine, alanine and asparagine it is called as Glycine, alanine and asparagines rich protein (Figure 6).

The matched peptide sequence is as follows:

LTSLVDASASARASASANAGGFGGSGAGGSGGNGFGGG
SGGSGFGGGSGGSGFGGGSGGSGFGGGSGGSGFGGGSG
GSGFGGGSGGSGFGGASASASAQALASATAELQAAQDA
YDQASAYAEATARVQVAAAAAAARAAASAASASASASAS
GSSFGSGGSGGSGNGGFGSFGASANAVANAFAQAFGGG
LGNGGNGGNGNGGNGGNGGNGNGGNGGNGNGGNGGN
GNGGNGGNGGNGNGGNGGNGNGGNGRAAASAASASA
SASASGSSFGSGGSGGSGNGGFGSFGASANAVANAFAQA
FGGGLGNGGNGGNGNGGNGGNGGNGNGGNGGNGNGG
NGGNGNGGNGGNGGNGNGGNGGNGNGGNGRNGNGGN
GR

Physicochemical property of identified sequence

The property of the sequence identified from AMP predictor revealed the charge of the sequence as negative charge and the hydrophobic ratio was 70%. As only very few anionic antibacterial peptides were reported, the identification of peptide fraction with negative charge from the whole tissue of *Donax cuneatus* is highly notable.

Table 1: Agar Well Diffusion Assay Of Crude Protein Extract

S.No	Pathogen	Zone of inhibition of crude extract 100µg/mL	Zone of inhibition of chloramphenicol 10µg/mL
1.	*Proteus vulgaris*	20.8 ± 0.7	5.3 ± 0.5
2.	*Klebsilla pneumoniaE*	16.3 ± 0.8	10.5 ± 0.5
3.	*Salmonella typhi*	15.3 ± 0.8	10.6 ± 0.5
4.	*Shigella flexneri*	15.1 ± 0.9	11.1 ± 0.7
5.	*Streptococcus pyogens*	14.6 ± 0.5	8.1 ± 0.7
6.	*Escherichia coli*	16.5 ± 0.5	14.5 ± 0.5
7.	*Bacillus subtilis*	22.3 ± 0.8	11.5 ± 0.5
8.	*Pseudomonas aeruginosa*	16.5 ± 0.5	10.5 ± 0.5
9.	*Staphylococcus aureus*	24.3 ± 0.8	10.5 ± 0.5

Table 2: MIC And MBC Assay Of Crude Protein Extract

S.No	Pathogen	MIC of crude protein extract (µg)	MBC of crude protein extract (µg)
1.	*Proteus vulgaris*	20	20
2.	*Klebsilla pneumonia*	10	10
3.	*Salmonella typhi*	10	10
4.	*Shigella flexneri*	10	NA
5.	*Streptococcus pyogenes*	10	NA
6.	*Escherichia coli*	20	NA
7.	*Bacillus subtilis*	10	NA
8.	*Pseudomonas aeruginosa*	20	NA
9.	*Staphylococcus aureus*	30	NA

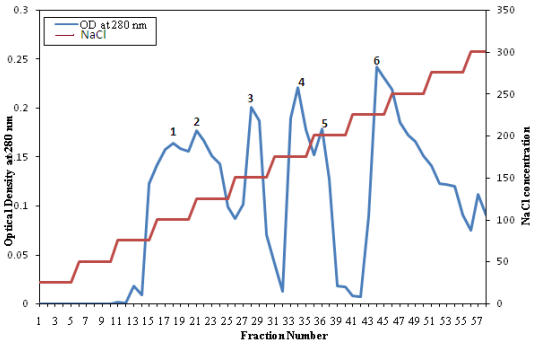
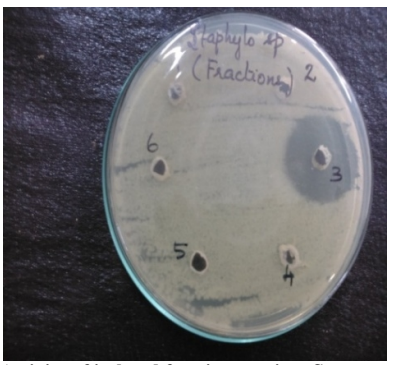


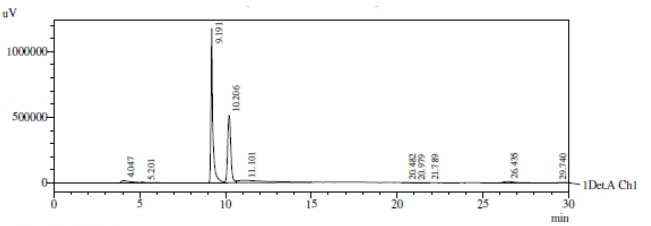
Figure 1: Elution profile of crude DC extract

Activity of isolated fractions against *P.aeruginosa*



Activity of isolated fractions against *S.aureus*

Figure 2: Activity of anion column fractions against *P.aeruginosa* and *S.aureus*



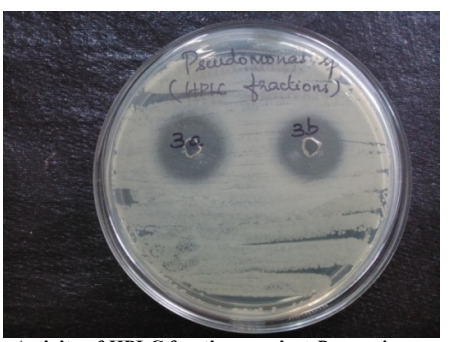
1 Det.A Ch1 / 230nm

PeakTable

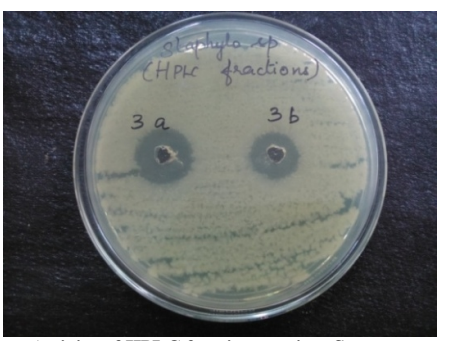
Detector A Ch1 230nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.047	740763	17790	3.837	1.017
2	5.201	196292	4141	1.017	0.237
3	9.191	8963667	1177621	46.431	67.336
4	10.206	6647008	514861	34.431	29.439
5	11.101	2192364	18746	11.356	1.072
6	20.482	13977	1202	0.072	0.069
7	20.979	24203	1263	0.125	0.072
8	21.789	449	66	0.002	0.004
9	26.435	483600	11481	2.505	0.657
10	29.740	43023	1712	0.223	0.098
Total		19305345	1748883	100.000	100.000

Figure 3: HPLC of bioactive fraction



Activity of HPLC fractions against *P.aeruginosa*



Activity of HPLC fractions against *S.aureus*

Figure 4: Activity of HPLC fractions against *P.aeruginosa* and *S.aureus*

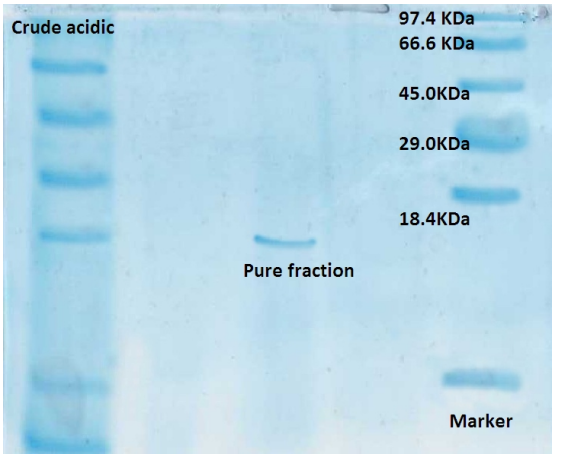
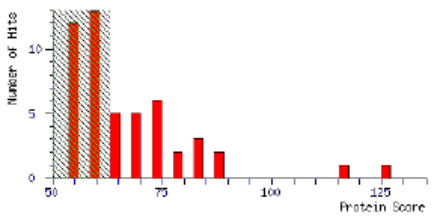


Figure 5: SDS-PAGE of the crude and pure fraction of *Donax cuneatus*
(A-crude protein extract, B-3rd fraction, C-marker)



Protein sequence coverage: 51%

Matched peptides shown in ***bold red***.

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  1 MLRVPLLVLC LALSVGADYY GYGWGRNGGG GGSGGGSGSS RASASASASA
 51 RANSIGNLVG RLTSLVDASA SARASASANA GGFGGSGAGG SGGNGFGGGS
101 GGSGFGGGSG GSGFGGGSGG SGFGGGSGGS GFGGGSGGSG FGGGSGGSGF
151 GGASASASAQ ALASATALQ AAQDAYDQAS AYAAATARAA ANGGSLDSSA
201 LASAIASAEA SVSARGASII ARARARAEAT VRAARRSFAS AQASAEASVS
251 AVRSAEGRAR SFARAVARAR ASARAAIAGV RSSGRAFASA TARARASVSA
301 AARAVARARA QAVARARASI RASASASARA SASAAAEARA AAYARVQVAA
351 AAAARAAASA ASASASASAS GSSFGSGGSG GSGNGGFGSF GASANAVANA
401 FAQAFGGGLG NGGNGGNGNG GNGGNGGNGN GGNGGNGNGG NGGNGNGGNG
451 GNGGNGNGGN GGNGNGGNGR NGNGGNGRNG NGGNGGNGNG RNGRGGRYYY
501 GSSDYYY
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Figure 6: Mascot search bar chromatogram of MALDI mass values and protein sequence

DISCUSSION

Consumption of bivalves in the regular diet is followed by very few coastal area people of our country. The inbuilt bioactive potential along with high nutritional content of these bivalves will greatly supports the health condition of the humans against various microbial born infections and other dangerous disorders. The antimicrobial peptides so far isolated also possess broad spectrum bioactivity like anticancer, anti-inflammatory, immunomodulatory effects etc. The crude and isolated peptide fraction of the edible bivalve was identified to possess antibacterial potency against both Gram positive and Gram negative bacteria. The study was also designed to prove the concept "Food as Medicine".

The Glycine, alanine and asparagine rich peptide fraction isolated with antibacterial property was reported for the first time. The protein was identified previously from *Haliotis asinina*, which was the membrane secretory protein with the function of shell formation in the species. From our study it is proved that the membrane secretory protein also possess a good antibacterial susceptibility.

The anionic peptides isolated from eukaryotic sources are generally aspartic-acid-rich molecules [23]. These antibacterial compounds are similar in action to the charge-neutralizing pro-peptides of serine proteases and they could regulate many of the body mechanisms of marine invertebrate. The antimicrobial peptide purified from the mollusc *Cenchritis muricatus* was also reported as anionic peptides [22].The antimicrobial peptides partially purified from the crab *Scylla serrata* showed high antimicrobial effect against the gram positive bacteria *Streptococcus pyogenes* [21].Many sponges of marine origin also act as good sources of antibacterial agents. The peptide polydiscamide A from the marine sponge *Discodermia sp* was found an efficient source against *Bacillus subtilis* [24].

The matched sequence was identified for their physico chemical properties from AMP predictor and the hydrophobic ratio of the sequence was 70 % which helps in binding with the bacterial surface and the protein binding potential according to Bomen index was 0.46 kcal mol^{-1} [20]. The net charge of the aforesaid matched sequence as per the predictor was approximately -1. The sequences rich in asparagine basically have negative charge hence the peptides are considered to be anionic. Generally, antimicrobial peptides have positive charge and are cationic in nature. Very few antimicrobial peptides of negative charge (anionic in nature) were reported [22] .The first reports of Anionic antimicrobial peptides / proteins were given in the early 1980s and they are identified to have important role in the innate immune systems of invertebrates as there is lack of acquired immune response. The peptides derived from them were found to have antimicrobial efficacy against many Gram positive and Gram negative bacteria, fungi and viruses [8]. The pure peptide with active property derived from *Donax cuneatus* was also of innate immune origin.

CONCLUSION

The crude and bioactive fractions of peptides isolated from the edible bivalve were observed to have potent antimicrobial

property. The activity was proved practically with antimicrobial assay technique and theoretically with the freely accessible web based tools. As the bivalve *Donax cuneatus* was considered to be protein rich edible source, the consumption of the meat in normal regular diet may help to improve the innate immune system of the body. Hence it might be a chance for development of body resistance against infectious diseases and it can also be developed into a better biopharmaceuticals for bacterial infections, in the future.

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