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Biomaterials of Marine Crustaceans – Prospects and Perspectives

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Abstract: *Food processing produces large quantities of by-products. Disposal of waste can lead to environmental and human health problems, yet often they can be turned into high value, useful products. Crustacean shell wastes from shrimp, crab, lobster, and krill contain large amounts of chitin, a polysaccharide that may be extracted after deproteinisation and demineralization of the exoskeletons .crustacean shellfish wastes its derivatives, have many biological activities (e.g., anti-cancer, antioxidant, and immune-enhancing) and can be used in various applications (e.g., medical, cosmetic, food, and textiles).This study explored various possible industrial applications of biomaterials available from Marine crustaceans. Biomaterial such as hemolymph and chitosan made from shell waste of Scylla serrata(mud crab) is been analysed for its potential antimicrobial ,anti-cancerous, and nanoparticle producing properties.*

Keywords:*Biomaterial , Chitin ,Hemolymph,Marine, Industrial application, Pharmaceutical Application*

I. Introduction

Modern food production generates a large quantity of by-products most of which are still underutilized. Yet, these food wastes may often contain several usable substances of high value including some that may have important health benefits. Generation of waste during the processing of food is unavoidable and disposal can be one of the major problems for those industries and for society. Especially if not done properly it can have negative impacts on the environment (i.e., pollution), create risks to human health, and a loss of income to the waste generator. For example, the seafood processing industry produces a large quantity of by-products and discards (heads, tails, skins, scales, viscera, backbones, and shells). These residual materials may be an excellent source of proteins, lipids, pigments, and small molecules like antimicrobial,anti-cancerous and immunomodulators. In addition the shell materials may be a source of chitinous materials

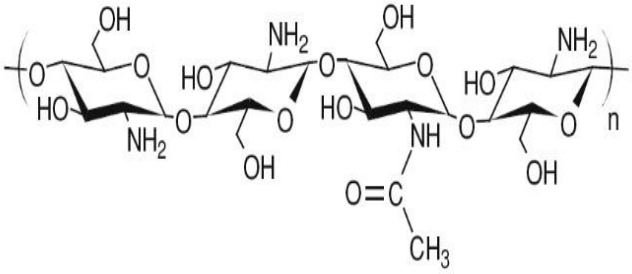
Crustaceans form a very large group of arthropods, usually treated as a subphylum, which includes familiar animals such as crabs, lobsters, crayfish, shrimp, krill and barnacles [1].*Scylla serrata*(often called mud crab or mangrove crab) is an economically important species of crab found in the estuaries and mangroves of Africa, Australia and Asia. In their most common form, the shell colour varies from a deep, mottled green to very dark brown.The natural range of *Scylla serrata* is in the Indo-Pacific. It is found from South Africa, around the coast of the Indian Ocean to the Malay Archipelago, as well as from southern Japan to south-eastern Australia, and as far east as Fiji and Samoa. The species has also been introduced to Hawaii and Florida[2&3].Interest in the aquaculture of this species has been high due to the high demand/price for them, high flesh content, and rapid growth rates in captivity[4].

Hemolymph, or hemolymph, is a fluid, analogous to the blood in vertebrates, that circulates in the interior of the arthropod body remaining in direct contact with the animal's tissues. It is composed of a fluid plasma in which hemolymph cells called hemocytes are suspended. In addition to hemocytes, the plasma also contains many chemicals. It is the major tissue type of the open circulatory system characteristic of arthropods[5].Hemolymph is composed of water, inorganicsalts (mostly sodium, chlorine, potassium, magnesium, and calcium), and organic compounds (mostly carbohydrates, proteins, and lipids). The primary oxygen transporter molecule is hemocyanin[6].Various studies on hemolymph of invertebrates are proved that hemolymph are the excellent source of Pharmacologically active materials and Immunomodulators.

The waste produced each year by the shellfish processing industries represent a practical challenge. With approximately 75% of the total weight of crustaceans (shrimp, crabs, prawns, lobster, and krill) ending up as by-products [8] and the current lack of acceptable waste management options there is a potentially large environmental hazard concern. Usually, seafood wastes are thrown away at sea, burned, landfilled, or simply left out to spoil.[9]Shell of crab is made up of chitin and the extraction of chitin from crustaceans' shells and its use as is or after further processing may be a way to minimize the waste and to produce valuable compounds with remarkable biological properties and crucial application in various fields.

There has been an interest with respect to better use of chitin, which is the second most available polysaccharide after cellulose. Chitin is obtained from crustaceans' exoskeletons after demineralization and deproteinisation treatments. However, one of the limitations in the use of this biopolymer on a large-scale is its water insolubility. Therefore, water-soluble derivatives have been produced. Chitosan is the most important of

these. It is obtained after deacetylation of chitin and it is theonly natural cationic polysaccharide known [10]. Chitin and its derivatives are renewable, biocompatible, biodegradable, and non-toxic compounds that have many biological properties such as: anti-cancer[11].,antioxidant [12]., anti-microbial [13] and anti-coagulant [14] properties. In addition, they are used as biomaterials in a wide range of applications: for biomedical purposes such as for artificial skin, bones, and cartilage regeneration [15] for food preservation such as for edible films [16], and for pharmaceutical purposes such as for drug delivery [17&18].



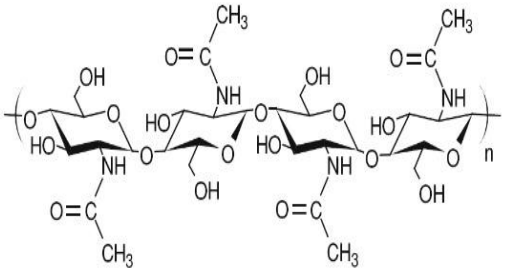


Fig 1 Structure Chitosan Chitin

Hence in the present study the possibility of various bioactive materials Crustaceans by products are being carried out. The study envisaged of collection of hemolymph and shell waste of *Scylla serrate or* mud crab and chitosan production from chitin.

II. Materials And Methods

Sample Collection and Preparation: Hemolymph wascollected by aspiration of hemolymph with a sterile syringe. To avoid hemocyte degranulationand coagulation, the hemolymph was collected in thepresence of sodium citrate buffer, pH 4.6 (2:1, V/V) Equal volume of physiological saline (0.85%, NaCl, w/v) was added to it. To remove hemocytes from thehemolymph it was centrifuged at 2000rpm for 15min at 4°C. Supernatant were collected by aspirating and stored at4°C until use.

Protein Estimation: Total protein was estimated (concentration ranges from0.5-1mg) using Lawry's method and spectrophotometric reading was done at 540nm against Bovine serum albumin as standard.

Antimicrobial Property: Antimicrobial activity was detected against different bacterial strains obtained from MTTC culture collection Pune.Inoculum was prepared from 1 days old cultures and suspensions were adjusted by using 0.5 McFarland standard.

FourierTransform-Infrared Spectroscopy (FT-IR)**:** FT-IRwas used to identify the chemical identities in a widerange of compounds. Infrared spectroscopy was a usefulanalytical technique for detection of functional groups inorganic compounds. Sample of 10 mg was mixed with 100mg of dried Potassium bromide (KBr) was compressed to prepare as a salt disc (10 mm) for spectrometer reading further. The infrared absorption bands identified molecular components and structures.

Determination of Molecular Weight**:** SDS-PAGE is used to find out the molecular weight active fractions of the sample. SDS-PAGE was performed in 12% separating gels, according to themethod described by Laemmli [19].

Chitin and Chitosan Extraction*:*The extraction of chitinand chitosan was performed according to the methodsdescribed by Arantus*et al.* [20] . In order to eliminate the proteins of theresidue, NaOH solutions 0.5M (30 : 1 v/m, 90∘C, 2 h) and0.3M(10 : 1 v/m, 80∘C, 1 h, under agitation), respectively,wereused.Then, the alkali-insoluble fraction (IFA) was separatedby centrifugation at 4000·*g* for 15 minutes and/or by vacuumfiltration. Subsequently, to demineralize the precipitate obtained, 10% acetic acid (100 : 1 v/m, 60◦C, 6 h) and 0.55Mhydrochloric acid (10 : 1 v/m, room temperature, 1,5 hours) were used. To obtain purified chitosan, treatments with 1%sulfuric acid (121∘C/20 min) and 50% NaOH (100◦C, 10 h)were performed.

Antioxident activity DPPH Assay :Chitosan (0.1-10mg/ml) in 0.2% acetic acid solution was mixed with 1ml of methanolic solution containing DPPH radicals in a final concentration of 10mM DPPH. The mixture was shaken vigorously and left to stand for 30min in the dark and the absorbance was then measured at 517nm against a blank. Ascorbic acid, BHA were used as standard. Scavenging ability (%) = [ΔA517 of control-ΔA517 of sample) /ΔA517 of control X100.

Nanoparticle synthesis**:**UV-visible spectroscopic data each sample was analyzed by UV-visible spectrophotometer (Systronics) in the range 200-750 nm and the wavelength corresponding to maximum absorption (λmax) was recorded. 0.1% (w/v) and 0.5% (w/v) chitosan in 1% (v/v) acetic acid solutions

III. Result

Hemolymph was isolated and the protein was estimated. Chitosan was synthesized and both biomaterial obtained was subjected to screened for its possible pharmacological applications.

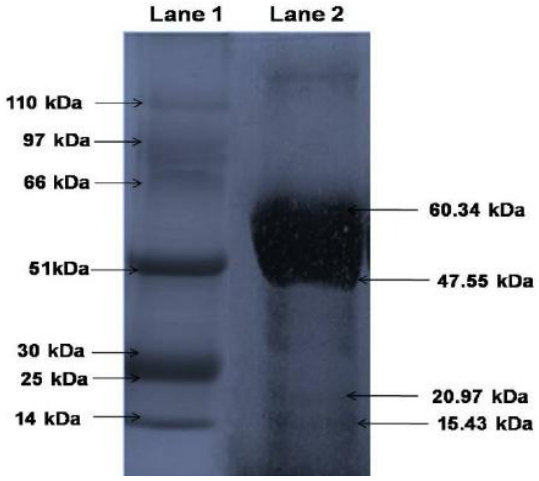


Fig1 SDS- PAGE Analysis of Hemolymph

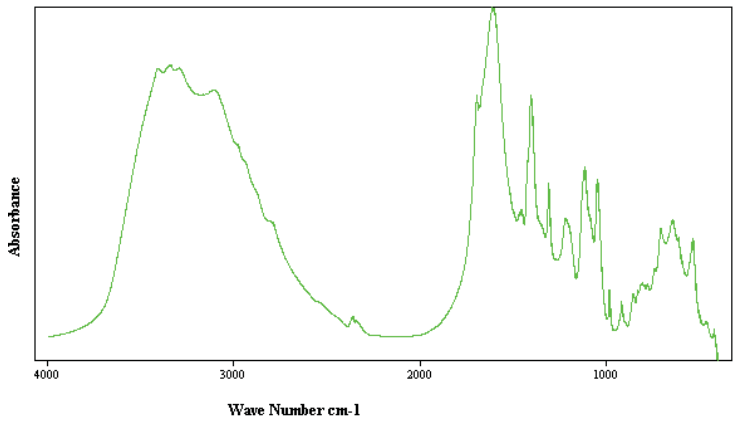


Fig2:FTIR analysis of Hemolymph

1. 3292 Amide A: Mainly N-H stretching of proteins ,2. 3084 Amide B: N-H stretching of proteins
3. 2871 CH3 symmetric stretch: ,4. 1656 Amide I: mainly C=O stretching of proteins ,5. 1536 Amide II: N-H bending and C-N stretching of proteins 6. 1394 COO- symmetric stretch: fatty acids and amino acids, 7. 1232 PO2 asymmetric stretch: mainly nucleic acids, 8. 1170 C-O-O-C asymmetric stretching: glycogen and nucleic acids ,9.1070 PO2 symmetric stretch: mainly nucleic acids

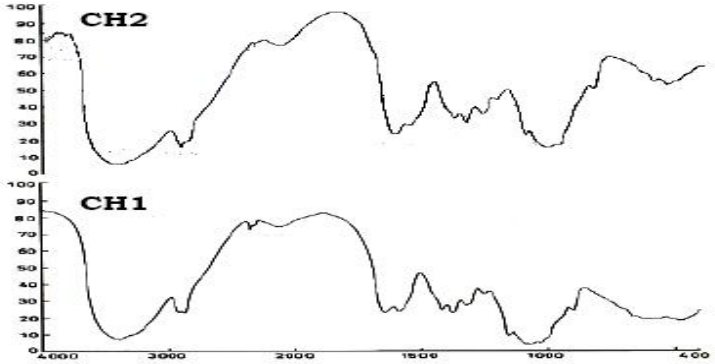


Fig3:FTIR Spectral Analysis Range of Chitosan1000-4000
Table 1: FTIR Analysis data of chitosan from crustacean

Chitosan Standard	**Chitosan Experimental**	**Vibration mode**
1.3423	3425	ν(NH2) assoc. in primary amines
2980	2985	ν (OH) assoc. in pyranose ring
1545	1550	ν (C=O) in NHCOCH3 group(Amide Group)
1130	1140	νas(C-O-C) (glycosidic linkage)
640	660	δ (NH) out of plane

Table2: Antimicrobial property of Hemolymph

Microorganism	Zone of inhibition 100µg/ml in (mm)		Control (mm) Antibiotic	
	100µl	200µl	100µl	200µl
Staphylococcus aureus	18±0.3mm	22±0.4mm	24±0.2mm	30±0.3 m
Streptococcus	16±0.3mm	18±0.3mm	24±0.2mm	24±0.2mm
E.coli	15±mm	17±mm	25±0.2mm	27.7±0.2mm
Pseudomonas fluorescence	10±0.2 mm	1.3±0.3mm	23± 0.4 mm	22±0.3mm

Table3: Antimicrobial Activity Chitosan

Microorganism	Zone of inhibition 100µg/ml in (mm)		Control (mm) Antibiotic	
	100µl	200µl	100µl	200µl
Candida albicans	15.5±0.3mm	17.5±0.4mm	24±0.2mm	27±3 mm
Staphylococcus aureus	19±0.3mm	21.±0.3mm	24±0.2mm	24±0.2mm
Streptococcus	194±mm	20.90±mm	25±0.2mm	277±0.2mm
E.coli	15±0.2 mm	22.1±0.3mm	23± 0.4 mm	25±0.3mm
Pseudomonas fluorescence	11±0.2 mm	18.1±0.3mm	23± 0.4 mm	25±0.3mm

Table4:DPPH Activity Chitosan

Samples	**Concentration of sample taken**	
Concentration of sample taken	50 mg ml–1	100 mg ml–1
	DPPH Activity%	
Chitosan	72%±1.1	81.3%±1.2
BHA	86.1%±1.3	95.3%±1.2
Ascorbic acid	84%±1.2	94.2%1.1

Fig4 Anticancerous properties of chitosan and hemolyph (A) Vero cell line (B) Hep 2 Cell line

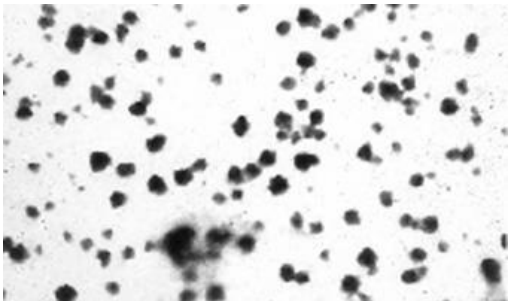


Fig 5:SEM analysis of Nanoparticle Synthesised from Chitosan

Hemolymph Protein Estimation: The protein content of the hemolymph was estimated using Lowry's method. Protein concentration of the hemolymph is measured using a spectrophotometer. The amount of protein present in the hemolymph was estimated as 2. 24mg/ml

SDS-PAGE :The hemolymph showed antibacterial activity was subjected to SDS-PAGE to estimate the molecular weight of proteins present in it. Different standard were used to determine the molecular weight of hemolymph proteins.The presence of bands were detected in the gel represents the presence of proteins in the range nearly 45- kDa

Spectral analysis**:** Asthe hemolymph sowed a good antimicrobial property a FT-IR spectral studies were carried out to screen for screening of basic functional compounds present in it. The results of FT-IR spectrum reveal that the hemolymph comprises to have peptide as their predominant chemical.

Antioxidant activity:The free radical scavenging activity was evaluated by using various in-vitro assays. DPPH radical was used as a substrate to evaluate the free radical scavenging activity of hemolymph extract. The scavenging effect of Chitosan on the DPPH radical was 72%±1.1 ($p<0.005$) and81.3%±1.2 at two different concentrations of 50 $ml^{-.1}$ and 100 mg $ml^{-.1}$

Chitosan synthesis:Amount of chitin obtained was between 70 and 250mg g−1 in different assays

Antimicrobial and Anticancerous activity:Anti cancerous study of hemolymph and Chitosan against Vero cell line and Hep2 cell line showed that it is a potent anticancerous agent. Antimicrobial study indicates that it showed a good antimicrobial effect against *Gram positive bacteria* .In this study, the exponentially grown Vero cells were treated with various concentrations of chitosan and chitosan nanoparticles ranging from 25 to 100 mg/mL, and the cell viability was measured by the MTT assay. The inhibition of cell viability by chitosan nanoparticles was clearly observed in a dose- and time-dependent manner

Nanoparticle synthesis: UV-visible spectroscopic data each sample was analyzed by UV-visible spectrophotometer (Systronics) in the range 200-750 nm and the wavelength corresponding to maximum absorption (λmax) was recorded. 0.1% (w/v) and 0.5% (w/v) chitosan in 1% (v/v) acetic acid solutions. Synthesis of Ag nanoparticle from Chitosan was confirmed by of SEM analysis

IV. Discussion

Search of new therapeutics and pharmacologically active compounds are the recent area of research in which great attention has been paid to study the bioactivity of natural products due to their potential pharmacological utilization. The present investigation is made on the basis of in search of antimicrobial peptides from the serum of hemolymph *Scylla serrata* of collected from the Vizhinjam ,Thiruvanathapram coastal environment. Hemolymph of *Scylla serrate* was found to be potent antimicrobial and anticancerous agent. Haug *et al.* [21] found the antibacterial activity in different body-parts of *Pagurus bernhardus* (Hermit crab), *Pandalus borealis* (Northern shrimp), *Hyas araneus* (Spider crab) and *Paralithodes camtschatica* (King crab). A similar result was observed with the hemolymph of some brachyuran crabs against clinical pathogens [12-25]. In the current examination hemolymph of *Scylla serrate* showed maximumantibacterial activity against *Staphylococcus aureus* (18mm). Li *et al*. [26] and induced *Bullacta exarata* with *E. coli*, isolated peptides from its mucus and lymph and found marked antimicrobial activity against E. coli and *S. aureus*. All these findings confirm the idea that endogenous antimicrobial peptides in marine invertebrates could be good resources for new antimicrobial compounds. This study corroborates a study on antibacterial activity against a variety of gram positive and gram negative bacteria of hemolymph of the shore crab called*Carinus maenas,* [27]. In the present investigation the percentage of protein concentration of hemolymph was recorded as 2. 24mg/ml. Similar type of study was carried out in various brachyuran crabs [18,23] and the concentration of protein in the hemolymph shows wide inter specific variation among decapod crustaceans, ranging from as low as 28mg/ml in *C. maenas* [24] to as high as 222mg/ml in *Uca minax* [25]. SDS PAGE analysis showed that a molecular weight ranges nearly 47.55 KDa-60.34 KDaprotein in *Scylla serrate* crab.Rameshkumar *et al.* [28] reported female crab *Charybdis lucifera* hemolymph and 25KDa proteins of male hemolymph crab of *C. lucifera*. A clear bands were detected in the gel which represents proteins of molecular weight 45KDa and 25KDa.These study also Suggested that the protein content in hemolymph has inter specific variations among crustaceans.

FTIR shows the possibility of overlapping N-H and O-H stretching vibrations, the strong band at 3000-3500 $cm^{-1}$and there is a characteristics of N-H stretching vibration observed[29].Silver nanoparticles were synthesised and controls $AgNO_3$ solution without chitosan showed no colour formation. This can be due to non-formation of AgNPs or instability of the synthesized AgNPs due to the absence of a stabilizer. This confirms the fact that the stabilizer plays a vital role in the synthesis of AgNPs. Also it is significant that higher the concentrations of AgNO3 and chitosan, higher the colour after irradiation. Antioxidant activity was found to be moderate when compared to standard. Yen *et al* (2008) reported the antioxidant activity Chitosan fromcrab shells.

The present study clearly indicates that the hemolymph of *Scylla serrate* crabwould be a good source of bioactive substance and would replace the existing in adequate and cost effective antibiotics. Antimicrobial peptides isolated from *Scylla serrate*would be a good source of bioactive substance and would replace the existing in adequate and cost effective antibiotics.

Chitosan synthesized from crab shell was found to be potent anti- cancerous and antifungal agent. Nanoparticle was synthesized from chitosan and was analysed through SEM and confirmed in identity. Marine by products are one the major resource of chitosan and was reported by many workers. Pharmacological potential of chitosan was also proved by many workers. In this study also confirmed that chitosan is an effective substance with various pharmacological properties.

In the present study indicated that crab waste materials such as Chitin and hemolymph are potential sources of biometerials having great bioprospecting potential .The pharmacology active material and industrially useful materials are obtained from these materials was found to be a potent antimicrobial anti cancerous and antioxidant agents. The chitosan obtained from the chitin of the crab can be used in the preparation of biofilm and antifungal agents. Hence the study report suggest that *Scylla serrate* rich marine resourceof novel compounds with great biological potential.

V. Conclusion

Study wasreached that crab waste is an excellent source of bioactive compound.. The yields and acetylation degree of chitosan decreased the concentration of NaOH solution, the temperature, and the length of treatment. The chitosan obtained showed the highest degree of deacetylation. Different chitosans were tested and markedly inhibited the growth of most bacteria tested; however, the inhibitory effects differed depending onthe types of chitosan and the bacteria tested, there beinggreater antimicrobial activity against Gram-positive bacteria than against Gram-negative bacteria.

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