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ORIGINAL ARTICLE

Antimicrobial activity of chitosan nanoparticles prepared from chitosan extracted from waste exoskeleton of *Litopenaeus vannamei* (Boon, 1931)

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ABSTRACT
In the present study, chitosan nanoparticles were prepared by ionic gelation method by mixing negatively charged tripolyphosphate (TPP) to positively charged chitosan extracted from the waste exoskeleton of shrimp Litopenaeus vannamei. SEM and XRD analysis was carried out for knowing the characteristics of chitosan nanoparticles. The antibacterial efficacy was tested on seven different pathogens by the disc diffusion method. SEM results showed that prepared chitosan nanoparticles have a smooth surface and homogeneous structure and XRD analysis confirms the crystalline nature of nanoparticles. It shows good antibacterial activity with the highest of 27mm zone of inhibition against E. coli and lowest zone of inhibition of 6 mm for Enterococcus faecalis, Klebsiella sp., and E. coli. The MIC values were recorded as 60, 60, 40, 40, 60, 80, and 40µl/ml for chitosan nanoparticle against tested bacterial strains of P. aeruginasa, E. faecalis, A. hydrophila, V. chloerae, Klebsiella sp., B. subtilis, and E. coli respectively. It can be concluded that the chitosan nanoparticles show the good results as an antibacterial agent for all the tested pathogens and the waste exoskeleton of shrimp from aquaculture pond can be made as an economically important product.
***Keyword*:-** *L.vannamei, Waste exoskeleton, Chitosan nanoparticles, SEM, XRD, Antibacterial activity.*

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INTRODUCTION

Nowadays the world is fast moving towards new technology in every field. It is predicted that in the future, nanotechnology will highly spread and get incorporated in every device, system, and material. In nanotechnology, nonmaterials have attracted many scientists, and the development of them is quite faster for their different applications. Study of nanomaterials has gained much more interest because of its uniqueness in size, stability, performance, size-dependent physical and chemical properties [1]. Maybe nanostructured biological probes are easier to make and are less expensive, will be more useful as a molecular probe [2]. Bio-detection of pathogens, drug and gene delivery, fluorescent biological labels, detection of proteins, probing of DNA structure, etc. are some of the applications of nonmaterials to medicine and biology. As proteins are an important part of the cells, so understanding their functions is very important. To identify the protein-protein interaction gold nanoparticles are used widely [3].

In the medical field, research for naturally available bioactive compounds is gaining more interest in the development of new medicine to overcome the use of synthetic medicine. Naturally available bioactive compounds are showing good results as antibacterial, antifungal, antioxidant, antiviral, etc. agents. As an antimicrobial agent, polysaccharide chitosan, which is composed of β-(1,4)- linked-D-glucosamine leftover with the amine groups has gained more interest [4]. Chitosan is natural, safe, biodegradable, biocompatible, harmless, cost-effective [5]. Helander [6] reported that radial growth, spore germination of fungus is easily prevented by chitosan. Nanoparticles showed antifungal activity which may be due to

its important properties such as particle size and zeta potential [7]. Other studies reported that the small size and quantum size effect of the chitosan nanoparticles may be the reason for their better biological activities.

Chitosan nanoparticles showed antimicrobial activity against some fungi and bacteria because of its unique nature. Chitosan nanoparticles loaded with insulin was found to increase intestinal absorption of insulin and decrease the serum glucose level of streptozotocin-induced diabetic rats by oral administration [8]. In drug-loading and tumor targeting therapy, the hydrotropic oligomer-conjugated glycol chitosan nanoparticles reported a remarkable anticancer effect after PTX encapsulation [9]. For cancer drug delivery as a carrier to deliver hydrophobic drugs like curcumin, dextran sulphate, chitosan nanoparticles can be used [10]. By conquering sporulation and spore formation, the chitosan nanoparticles exert an antifungal effect [11]. Mostly accepted antimicrobial activity of chitosan is that the negatively charged bacterial cell wall gets bind with chitosan and creates a disruption of the cell and changes the membrane permeability and gets attached to DNA that results in cell death by causing inhibition of DNA replication [12]. Chitosan takes action as a chelating agent in which trace metal elements get binds, which cause toxin production and holds bacterial growth [13].

The present study is done to test the efficacy of chitosan nanoparticles, as an antibacterial agent, prepared from chitosan extracted from the waste exoskeleton of shrimp *L.vannamei*.

MATERIALS AND METHODS

Preparation of chitin and chitosan

Extraction of chitin from the waste exoskeleton of shrimp *L. vannamei* was done by deproteinization and demineralization process and chitosan was prepared through the deacetylation process, following the method described by Hong and Samuel [14] with little modification. The exoskeleton of shrimp was collected from the aquaculture pond, extra flesh, waste material was removed manually and washed thoroughly first by tap water, and then by distilled water. Later these were dried in a hot air oven and powdered by using a mixer grinder. The powdered sample was deproteinized by treating it with 1N NaOH at 65^{o}C for 4-6 hours at the ration of 1:10 w/v. Then the sample was filtered and was with distilled water till the pH reaches neutral. Demineralization was done by treating the obtained sample by 1N HCl for 10-12 hours at room temperature at the ration of 1:10 w/v. The sample was washed with distilled water until the pH reaches neutral. The obtained sample was kept in ethanol for removing colored pigment, washed and dried to obtain chitin. Chitosan was obtained by the deacetylation process by treating chitin with 50% NaOH at 100^{o}C for 5-6 hours and cooled at room temperature. Washed with distilled water till pH reaches to neutral and freeze-dried.

Preparation of Chitosan nanoparticles

The chitosan nanoparticles were prepared by ionic gelation method at room temperature on the interaction between mixing of negatively charged tripolyphosphate (TPP) solution to positively charged chitosan solution as described by the Megha [15]. Chitosan was dissolved in a 1% acetic acid solution using a magnetic stirrer at room temperature till a clear solution is obtained. Surfactant tween 80 (0.5% v/v) was added to the solution for preventing particle aggregation. The pH of chitosan solution was raised to 4.6 - 4.8 with 1N NaOH. 10mg of sodium tripolyphosphate(TPP) was dissolved in 10ml of distilled water for preparing TPP solution. TPP solution was added to chitosan solution in a dropwise manner and mixed using a magnetic stirrer at 800 rpm at room temperature in the ration of 2.5:1 (Chitosan: TPP). The chitosan particle was centrifuge at 12,000g for 30 min. The pellet resuspended in water and chitosan nanoparticle suspension was freeze-dried.

Characterization of Chitosan nanoparticles-SEM

The structure of prepared chitosan nanoparticles was observed by using a scanning electron microscope (JEOL-JSM 5610LV, JEOL, Ltd, Tokyo, Japan) at an acceleration voltage of 10kV and a current of 10mA after sputter coating the samples with gold.

X-ray diffraction spectroscopy

X-ray diffractometer, Model-D8 Advance (Bruker, Germany) was used to study the chitosan nanoparticles' diffraction planes. CuK alpha target has been used with a wavelength of 1.54 □ and the operation of the generator with 45 kV and 30 mA current were performed.

Antibacterial activity

Seven human pathogens were selected for the present study, namely *Pseudomonas aeruginasa, E. coli, Aeromonashydrophila, Bacillus substilis, Enterococus faecalis, Klebsiella sp., Vibro cholera* and all strains were individually inoculated in the sterilized nutrient broth and incubated at 37^{o}C for 24 hrs.

The antibacterial activity of chitosan nanoparticles was carried out by using disc diffusion method. A stock solution was made by dissolving 5 mg/ml of chitosan nanoparticles in 0.25% acetic acid. A

suspension of tested microorganisms was spread on MH agar medium plate and sterile discs of 5 mm made by Whatman No. 1 filter paper were individually placed in the different concentrations (25, 50, 75 and 100 %) of chitosan nanoparticles and then placed in the agar plates. The plates were incubated at 37°C for 24hrs and the inhibition ring was noted by measuring the diameter of zone of inhibition in mm. Ampicillin 1 mg/ml was used as a positive control and 0.25% acetic acid was used as a negative control.

Minimum Inhibitory Concentration of the sample

MIC of chitosan nanoparticles was carried out by following the method of Seedevi [16]. A stock solution of 100 µg/ml was prepared and serially diluted to obtain a various range of concentrations between 20 µg/ml and 100 µg/ml. In a test tube, 0.5 ml of dilution containing 2 ml of nutrient broth was taken and 50µl of bacterial culture was inoculated. Ampicillin was used as positive control. All the test tubes were incubated at 37°C for 24hr. The test tube containing the least concentration of extract which is showing no visible sign of growth was taken as minimum inhibitory concentration.

RESULTS

The total yield of chitin and chitosan from the exoskeleton of *L. vannamei* was found to be 31.25% and 19.7% respectively, with 79.68% degree of deacetylation of chitosan.

Scanning Electron Microscope

The chitosan nanoparticles prepared by the ionic gelation method showed smooth surface and homogeneous structure fig (1) in the SEM image.

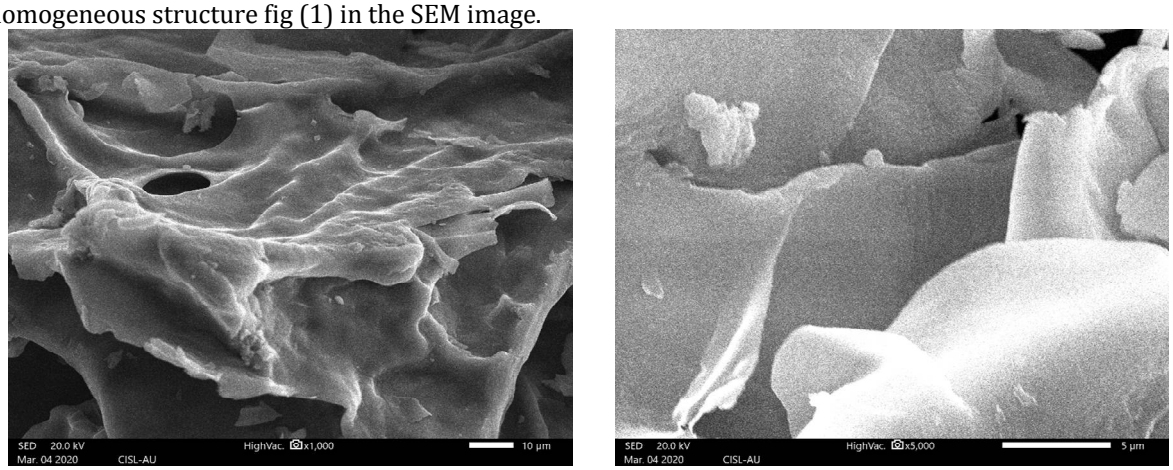


Fig 1:- SEM image for chitosan nonoparticles

X-ray diffraction spectroscopy

In the XRD pattern of chitosan nanoparticles, 2-theta degree was found at X=23.794 which possesses better crystallinity and the intensity was found at Y=611.089 (Fig 2).

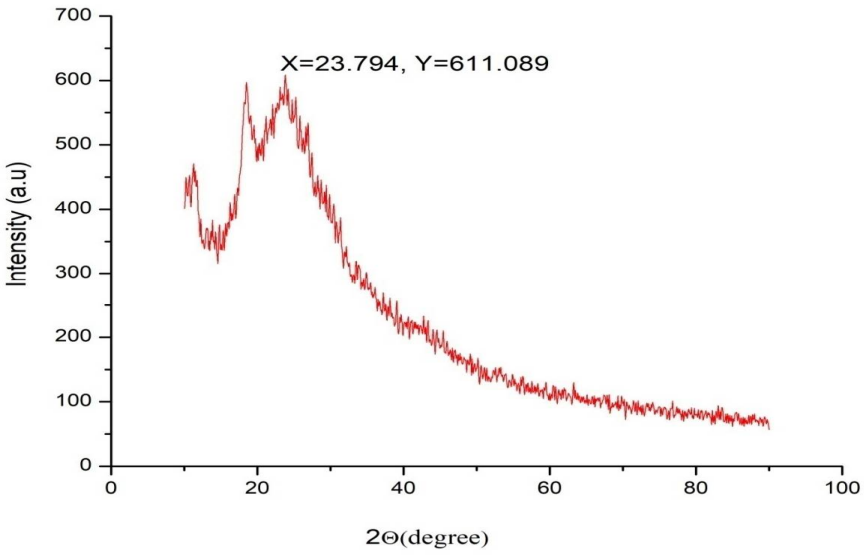


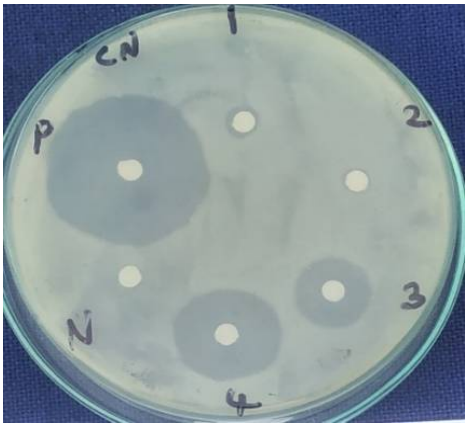
Fig 2: XRD pattern for chitosan nanoparticles

Antibacterial activity

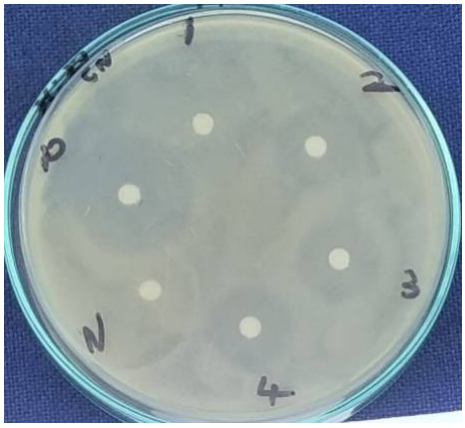
The chitosan nanoparticles showed antibacterial activity against all the 7 tested bacterial strains. In 100% concentration, chitosan nanoparticles showed a maximum of 22 mm of inhibition zone against *E. coli* and the lowest inhibition zone of 7 mm was observed against *E.faecalis*. In the case of 75% concentration, chitosan nanoparticles showed the highest activity with 27 mm against *E.coli*, whereas the lowest activity of 7 mm of inhibition zone, against *E. faecalis*. At 50% concentration, the chitosan nanoparticle showed a maximum inhibition zone of 18 mm against *E. coli* and *A.hydrophila*, on the other hand, the lowest zone of inhibition of 6 mm was seen in *E. faecalis, Klebsiella sp*. and *B. subtilis*. In 25% concentration, the chitosan nanoparticles showed 8 mm of inhibition zone against *B. subtilis* and the lowest of 6 mm was recorded against *E. faecalis*, *Klebsiella sp.* and *E. coli*. At the same time, the positive control Ampicillin showed 24, 26, 30, 31, 26, 26 and 27 mm of inhibition zone against all seven tested bacterial strain *P.aeruginasa, E. faecalis, A. hydrophila, V.chloerae, Klebsiellasp, B. subtilis* and *E. coli* respectively and the negative control disc reported no activity at all.

Table 1: Zone of Inhibition (mm) for chitosan nanoparticles

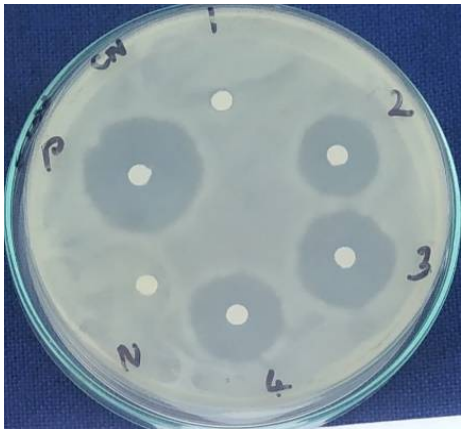
Sr. No.	Name of strain	25%	50%	75%	100%	+ve Control	-ve Control
1	*Pseudomonas aeruginasa*	-	16	16	16	24	-
2	*Enterococcus faecalis*	6	6	7	7	26	-
3	*Aeromonas hydrophila*	7	18	18	18	30	-
4	*Vibro chloerae*	7	7	15	19	31	-
5	*Klebsiella sp*	6	6	16	16	26	-
6	*Bacillus subtilis*	8	6	8	8	26	-
7	*E. coli*	6	18	27	22	27	-



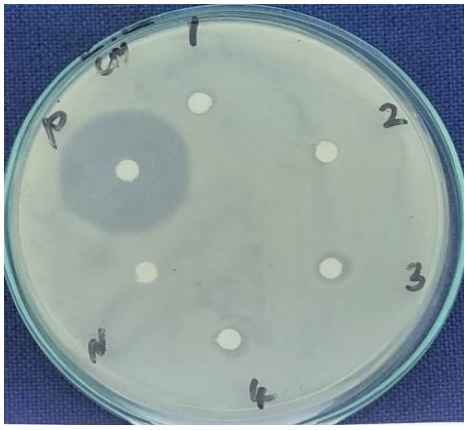
Vibro chloerae



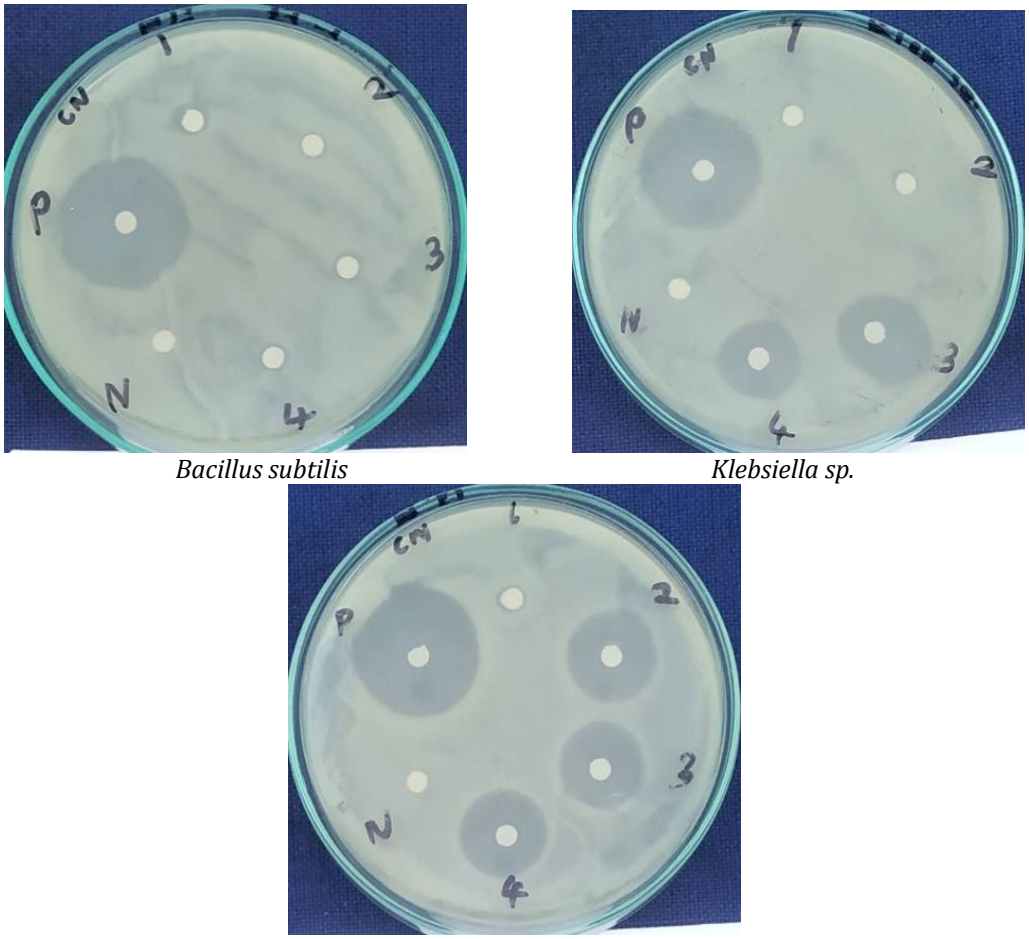
Pseudomonas aeruginasa



E. coli



Enterococcus faecalis



Bacillus subtilis *Klebsiella sp.*

Aeromonas hydrophila

Fig 3:- Antibacterial Activity of chitosan nanoparticles

Minimum Inhibitory Concentration

The MIC values for chitosan nanoparticles against tested sbacterial strains of *P. aeruginasa, E. faecalis, A. hydrophila, V. chloerae, Klebsiella sp., B. subtilis* and *E. coli* were recorded as 60, 60, 40, 40, 60, 80 and 40 µl/ml respectively (Table 2)

Table 2: MIC for chitosan nanoparticles

Name of strain	20 µl/ml	40 µl/ml	60 µl/ml	80 µl/ml	100 µl/ml	+ve control	-ve control
Pseudomonas aeruginasa	++	++	+-	+-	+-	--	++
Enterococcus faecalis	++	++	+-	+-	+-	--	++
Aeromonas hydrophila	++	+-	--	--	--	--	++
Vibro chloerae	++	+-	+-	+-	--	--	++
Klebsiella sp	++	++	+-	--	--	--	++
Bacillus subtilis	++	++	++	+-	--	--	++
E. coli	++	+-	--	--	--	--	++

MIC: -- No growth , +- Little turbid solution , ++ Fully Turbid Solution

DISCUSSION

Chitin is one of the polysaccharides available in large quantities in many living organisms, consists of 2—acetamido-2-deoxy-β-D-glucose through a β(1→4) linkage, present in the external shell of crustaceans, internal bone of mollusks, fungi [17; 18; 19]. Chitin is a white, hard nitrogenous polysaccharide with biodegradability and bioactive properties, a novel type of functional compound [20]. The yield of extracted chitin varies from different sources. The average percentage of chitin content from shrimp was 30-40% [21]. From different crustaceans the percentage yield of chitin was 21.26% from lobster *Thenus orientalis,* 16.75% from shrimp *Metapenaeus affinis,* 20.60% from crayfish shells, 21.53% from brown shrimp *Penaeus aztecus,* 23.72% from shrimp *Penaeus durarum,* 19.13% from tiger prawn *Penaeus*

semisulcatus, 20.8% from male crab *Portunus pelagicus* and 20.14% from female crab of the same species [22; 23]. Shanmugam [24] calculated 11.96% yield of chitin from the shell of bivalve donacid clam *Donax scortum*. In the present study the yield of chitin from exoskeleton of *L. vannamei* was found to be 31.25%, which is slightly higher than that of the majority of the crustaceans cited above.

Chitosan is a non-toxic and biocompatible cationic polysaccharide produced by partial deacetylation of chitin and composed of glucosamine and N-acetylglucosamine. Because of various properties of chitosan such as lack of toxicity, its biocompatibility, biodegradability, bioactivity, etc., it exhibits numerous application in the field of wastewater treatment, agriculture, fabric and textiles, cosmetics, nutritional enhancement, food processing and pharmaceuticals. The usefulness of chitosan as a wound healing accelerator and its effectiveness in protecting wounds from bacterial invasion by suppressing bacterial proliferation and its effectiveness against typhoid producing microorganisms was well studied by Yadav and Bhise [25].

From the shell and operculum of *N. crepidularia* the yield of chitosan was found to be 31.14% and 44.29% respectively [26]. Danarto and Sperisa [27] reported that chitosan yield in green mussel shell was 39.5%. Shanmugam [24] recorded the yield of chitosan from *D. scortum* as 18.8%. In the present study the yield of chitosan is 19.7% from the exoskeleton of *L. vannamei* which is nearly equal to the yield of chitosan from *D. scortum* Shanmugam et al. [24] and lesser than from green mussel shell [28].

In pharmaceuticals or for drug release the morphology of the compound is supposed to be important [27]. The morphology and structure of the prepared sample are mostly analyzed by the SEM images. Previously, the morphology of the chitosan nanoparticles prepared by ionic gelation method was studied by SEM analysis that showed homogeneous structure and spherical shape [15]. In the present study, the SEM analysis for prepared chitosan nanoparticles, shows the somewhat same result as previous studies whose surface is also smooth and has homogeneous structure.

Observing the XRD pattern of the chitosan derivatives, having amorphous forms indicates that they may be useful in biomedical applications [29]. In the present study, the XRD pattern of the chitosan nanoparticles shows 2-theta degree X=23.794 which possess better crystallinity and the intensity was found at Y=611.089, the intensity is minimum for the chitosan nanoparticle in this study.

Old research shows chitosan nanoparticles have good antibacterial activity for gram negative as well as for gram positive bacteria. A previous study concluded that chitosan and chitin are less active than chitosan nanoparticles [13]. Chitosan nanoparticles prepared by ionic gelation of chitosan with tripolyphosphate anions, show that it can inhibit the growth of bacteria tested [30]. Superior antimicrobial activity of chitosan nanoparticles was seen against medical pathogens such as *K. pneumoniae*, *E. coli, S. aureus and P. aeruginosa,* when compared to that of chitosan and chitin [13]. Chitosan nanoparticles prepared using Tripolyphosphate by ionotropic gelation method showed good antimycobacterial effect for *M. tuberculosis* H37Rv [31]. In the present study, chitosan nanoparticles prepared using chitosan extracted from waste exoskeleton of shrimp *L. vannamei* shows antibacterial activity against all tested pathogens in concentration dependent manner.

A previous study reported that in bacterial suspension, chitosan nanoparticles can be well distributed and to the surface of chitosan nanoparticles bacteria can easily adhere in a short time thus chitosan nanoparticles showed antibacterial activity [30]. The growth of microorganisms is suppressed or they get killed by the release of intracellular material (nucleic acid) from microorganisms treated with chitosan nanoparticles [13]. Bacterial cell wall coated with chitosan nanoparticles causes destruction, condensed masses of a bacterial cell wall, leakage of cytosolic components from bacteria forces the bacteria to die [32].

The present study not only shows the antibacterial activity of chitosan nanoparticles but also shows the utilization of the waste exoskeleton of shrimp for pharmaceutical importance. So the shrimp exoskeleton thrown as waste can be utilized as the raw material for the extraction of the natural biopolymer, the Chitin which in turn forms the basis for the chitosan nanoparticles.

CONCLUSION

Chitosan nanoparticles show the efficacy as an antibacterial agent because of their unique character and structure. The chitosan nanoparticles by ionic gelation method, from chitosan extracted from exoskeleton of *L. vannamei* show the good result as an antibacterial agent for all the tested pathogens. The SEM results show the homogenous structure and smooth surface; whereas XRD result shows that the chitosan nanoparticles are crystal in nature. Overall it can be concluded that the waste exoskeleton of shrimp from aquaculture pond can be made as an economically important product and will be useful for reducing the waste, which will help to keep the environment clean.

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