



Preparation, characterization and antibacterial activity of chitosan and phosphorylated chitosan from cuttlebone of *Sepia kobsiensis* (Hoyle, 1885)



Annaian Shanmugam^{a,*}, Kandasamy Kathiresan^a, Lakshman Nayak^b

^a Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu 608 502, India

^b Department of Marine Sciences, Berhampur University, Berhampur, Odisha 760007, India

ARTICLE INFO

Article history:

Received 29 April 2015

Received in revised form 24 September 2015

Accepted 17 October 2015

Available online 10 November 2015

Keywords:

Cephalopod

Chitin

Chitosan

Water soluble

Phosphated chitosan

ABSTRACT

Chitosan is a commercially available derivative of chitin that has been extensively studied for its antimicrobial properties. In order to improve the water solubility and its biological activity, the chemical modification or derivatisation is attempted. In the present investigation, the chitosan prepared from the cuttlebone of *Sepia kobsiensis* was being chemically modified by reacting it with orthophosphoric acid so as to obtain phosphorylated chitosan. Then the chitosan and phosphorylated chitosan were structurally characterized through FT-IR spectroscopy. Further the antibacterial activity of chitosan and phosphorylated chitosan was tested against clinically isolated human pathogens (Gram-positive: *Streptococcus* sp., *Streptococcus pneumoniae* and *Staphylococcus aureus* and Gram-negative: *Escherichia coli*, *Vibrio cholerae*, *V. alginolyticus*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* sp. and *Proteus vulgaris*) by well diffusion method and the Minimum Inhibitory Concentration (MIC) was also calculated. The results of the present study suggests that the chitosan and phosphorylated chitosan has concentration dependent antibacterial activity with variation against several pathogenic human pathogenic bacterial strains which indicates their possible use as antibacterial agents.

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1. Introduction

The Class: Cephalopoda which includes the Nautilus, cuttlefishes, squids and octopods, is the most advanced class of the Phylum: Mollusca adapted to a swimming existence. They are exclusively marine, diverse in form, size and nature [1], and occupy littoral and benthic to pelagic environments of all the oceans. On Earth, chitosan is the second most abundant polysaccharide next to cellulose. As a natural renewable resource, chitosan has a number of unique properties such as antimicrobial activity, non-toxicity and biodegradability, which attract scientific and industrial interest in the fields such as biotechnology, pharmaceuticals, wastewater treatment, cosmetics, agriculture, food science and textiles [2,3].

Chitosan has a wide inhibition spectrum for not only Gram-positive and Gram-negative bacteria but also yeast and moulds. The poor solubility of chitosan has limited its application as a polysaccharide drug. In order to improve its solubility and

bioactive potential, the chitosan derivative had been prepared by incorporating sulfate, phosphate, carboxyl group etc. [4–7] and their biological activities such as antioxidant activity, antibacterial activity, anticoagulant activity have also been studied by several researchers [8]. Subhapradha et al. has prepared phosphorylated chitosan from the gladius of *Sepioteuthis lessoniana* from Thondi coast and studied its *in vitro* antioxidant activity [4]. Likewise Barwin Vino et al. has extracted, characterized and studied the *in vitro* antioxidant potential of chitosan and sulfated chitosan from the cuttlebone of *Sepia aculeata* collected from Cuddalore coast [5]. Further Ngo et al. extensively reviewed the biological effects of chitosan and its derivatives. There is an ever continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action due to the alarming increase that has been witnessed in the incidence of both new and reemerging infectious diseases. A further big concern is the development of resistance to the antibiotics in current clinical use [9,3]. The purpose of this investigation was to evaluate the antibacterial potential of chitosan and its water soluble derivative, phosphorylated chitosan, prepared from the cuttlebone of cuttlefish *Sepia kobsiensis*.

* Corresponding author. Fax: +1 4144 243555.

E-mail address: shanpappu48@gmail.com (A. Shanmugam).

2. Materials and methods

2.1. Collection of animals

The cuttlebones were removed from *S. kobsiensis* purchased from Mudasalodai (Cuddalore, Tamil Nadu) landing center (lat. 11° 50' N; long. 79° 77' E) during the period of March–April 2015. The cuttlebones were washed thoroughly in tap water followed with distilled water. They were then dried and pulverized using pestle and mortar for further analysis.

2.2. Extraction of chitin and chitosan

Chitin was extracted from the cuttlebone of *Sepia kobsiensis* and a chitosan with DDA of 85.55% and molecular weight of 322.04 kDa was prepared [10].

2.3. Preparation of phosphorylated chitosan

Phosphorylated chitosan was prepared by dissolving 2 g of chitosan powder with 30 g of urea and 50 ml of *N,N*-dimethylformamide (DMF) followed which 5.2 ml of orthophosphoric acid was added and the mixture was allowed to react at 150 °C for 1 h. After cooling, the reaction mixture was precipitated and washed thoroughly with methanol and then the residue was re-dissolved in distilled water. The pH was adjusted to 10– 11 by sodium hydroxide. The solution was dialyzed against distilled water for 48 h using a 12 000 Da MW cut-off dialysis membrane. Then the product was lyophilized to get phosphorylated chitosan.

2.4. FT-IR spectral analysis of chitosan and phosphorylated chitosan

FT-IR spectroscopy of solid samples of chitosan and phosphorylated chitosan obtained from the cuttlebone of *S. kobsiensis* were relied on an AVATAR 330 FT-IR spectrometer. 10 µg of each sample was mixed with 100 µg of dried potassium bromide (KBr) and compressed to prepare salt disc (10 mm diameter) for reading the spectrum further.

2.5. Determination of antibacterial activity by agar well diffusion method

Eleven species of bacteria (clinical isolates) (Gram-positive: *Streptococcus* sp., *S. pneumoniae* and *Staphylococcus aureus*; Gram-negative: *E. coli*, *Vibrio cholerae*, *V. alginolyticus*, *V. parahaemolyticus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* sp. and *Proteus vulgaris*) obtained from the Raja Muthaiah Medical College Hospital, Annamalai University, Annamalai Nagar, Tamil Nadu, India were used as test organisms. Nutrient broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. Individual species of bacteria were inoculated in the sterile nutrient broth and incubated at 37 °C for 24 h. Mueller Hinton Agar (MHA, Himedia) medium was prepared, sterilized in an autoclave at 15 lbs pressure for 15 min and poured into sterile petridishes and incubated at 37 °C for 24 h. The antibacterial activity of the individual bacterial strains was tested using Agar well diffusion method [9]. 24 h old nutrient broth cultures of test bacteria were aseptically swabbed on sterile nutrient agar plates. Wells of 5 mm diameter were made aseptically in the inoculated plates. The different concentrations of chitosan (Stock–5 mg/ml in 0.2% Acetic acid) and phosphorylated chitosan (Stock–5 mg/ml in distilled water), from this stock solution four different concentrations viz., 25, 50, 75 and 100% prepared were loaded in the respective wells. Standard (Tetracycline, 1 mg/ml) and Control (0.2% acetic acid) were also loaded into the respectively labeled wells. The plates were incubated at 37 °C for 24 h in upright position. The experiment was carried out in triplicate and the zone of inhibition was recorded.

2.6. Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of chitosan and its derivative phosphorylated chitosan was determined by standard method [11]. In this method, a stock solution of 100 µg/ml was prepared. This was serially diluted to obtain various concentrations lying between 5 µg and 100 µg/ml. 0.5 ml of each of the dilutions of different concentrations was transferred into sterile test tube containing 2 ml of nutrient broth. To the test tubes, 0.5 ml of test organisms previously adjusted to a concentration of 10⁵ cells/ml was then introduced. A set of test

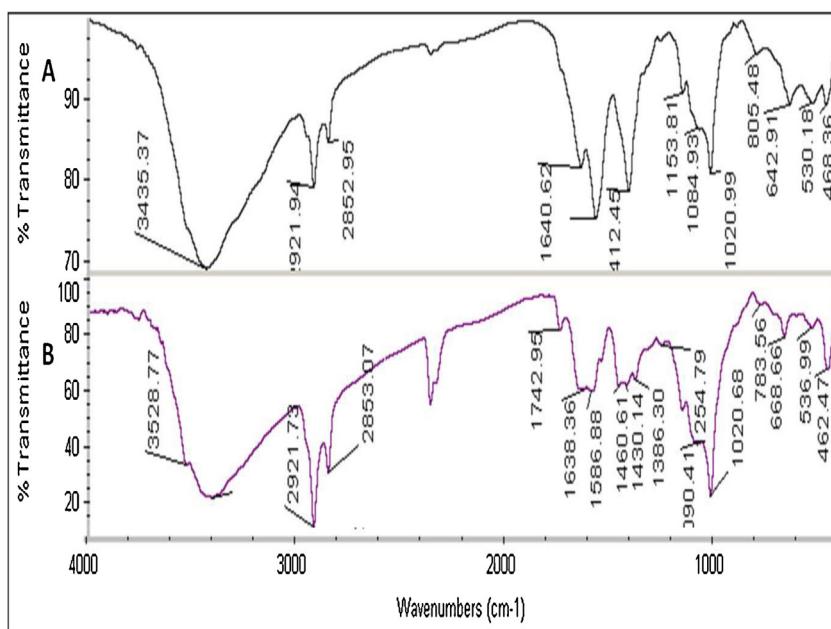


Fig. 1. FT-IR spectrum of chitosan (A) and phosphorylated chitosan (B) from *Sepia kobsiensis*.

tubes containing broth alone were used as control. All the test tubes and control were then incubated at 37 °C for 18 h. The tubes were then studied for the visible signs of growth or turbidity after the period of incubation. The lowest concentration of chitosan and phosphorylated chitosan that inhibited the growth of bacteria was considered as the Minimum Inhibitory Concentration.

2.7. Statistical analysis

Data obtained from the experiments (in triplicate) on the inhibitory effects of chitosan and phosphorylated chitosan were analyzed by one-way analysis of variance (ANOVA) using SPSS software (16 version) followed by Duncun's multiple range test (DMRT) and standard error \pm . 'P' values at <0.05 were considered for describing the significant levels.

3. Results

3.1. FT-IR spectroscopy

The FT-IR spectra of chitosan and phosphorylated chitosan are depicted in Fig. 1A&B, respectively. The peak at 3435.37 cm^{-1} corresponds to H-bonded NH_2 and OH stretching. The peaks at 2921.94 cm^{-1} and 2852.95 cm^{-1} correspond to aliphatic CH stretching. The peak obtained at 1640.62 cm^{-1} corresponds to the amide stretching of C=O and the band at 1020.99 cm^{-1} attributes to C—O—C stretching vibrations. In phosphorylated chitosan the peak found at 1386.30 cm^{-1} attributes to P=O stretching which confirms the phosphorylation.

3.2. Antibacterial activity

The chitosan and phosphorylated chitosan from *S. kobeensis* showed good antibacterial activity against almost all pathogenic bacteria (Figs. 2 and 3). The antibacterial activity was found to be concentration dependent. At the same time the activity was absent in negative control (Table 1). In 100% concentration, the highest inhibition zone of 17 mm diameter clear zone was noticed against *P. vulgaris* in chitosan (Fig. 2) and 16 mm clear zone recorded against *Staphylococcus aureus* in phosphorylated chitosan (Fig. 3). The lowest inhibition zone of 13 mm 7 mm was observed against *S. pneumoniae* in chitosan and *Proteus vulgaris* in phosphorylated chitosan respectively. In 75% concentration, chitosan showed highest activity of 15 mm inhibition zone against *P. vulgaris*, *P. aeruginosa*; whereas phosphorylated chitosan showed 12 mm in *S. aureus*. The lowest activity with 12 mm inhibition zone was observed against *K. pneumoniae* and *E. coli* in chitosan and 7 mm against *Salmonella* sp. in phosphorylated chitosan.

In 50% concentration, the maximum activity of 14 mm was recorded against *P. vulgaris* and *P. aeruginosa* in chitosan and 9 mm against *S. aureus* in phosphorylated chitosan. The lowest 10 mm inhibition zone against *S. pneumoniae* in chitosan; whereas phosphorylated chitosan reported no activity in all the bacterial strains tested except *Staphylococcus aureus*. In 25% concentration, the maximum activity of 13 mm was recorded against *P. vulgaris*, *P. aeruginosa* in chitosan and 7 mm in phosphorylated chitosan against *S. aureus*. The lowest 9 mm against *S. pneumoniae* in chitosan and in phosphorylated chitosan minimum of 7 mm against *Staphylococcus aureus* and no activity was noticed in all other strains. The positive control tetra cycline showed the

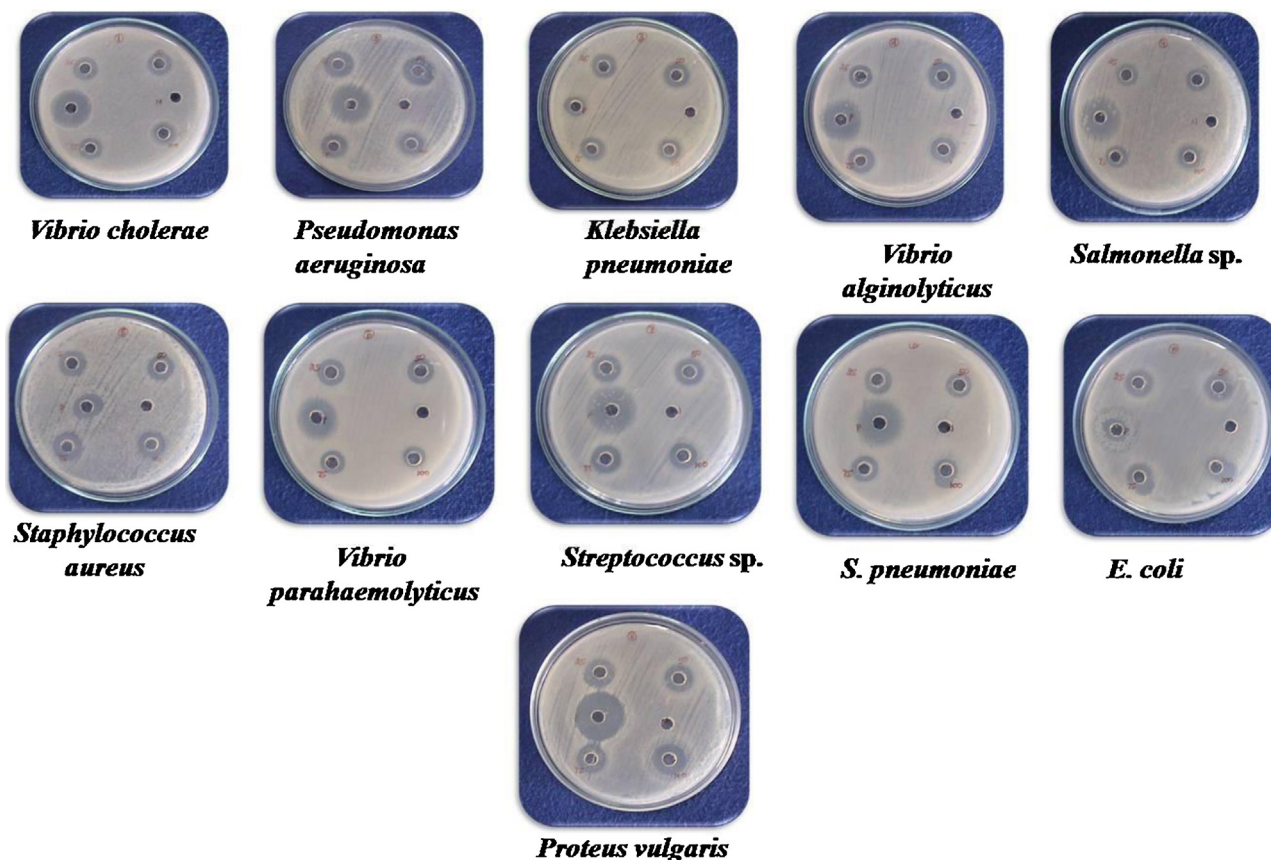


Fig. 2. Antibacterial activity of chitosan from *S. kobeensis*.

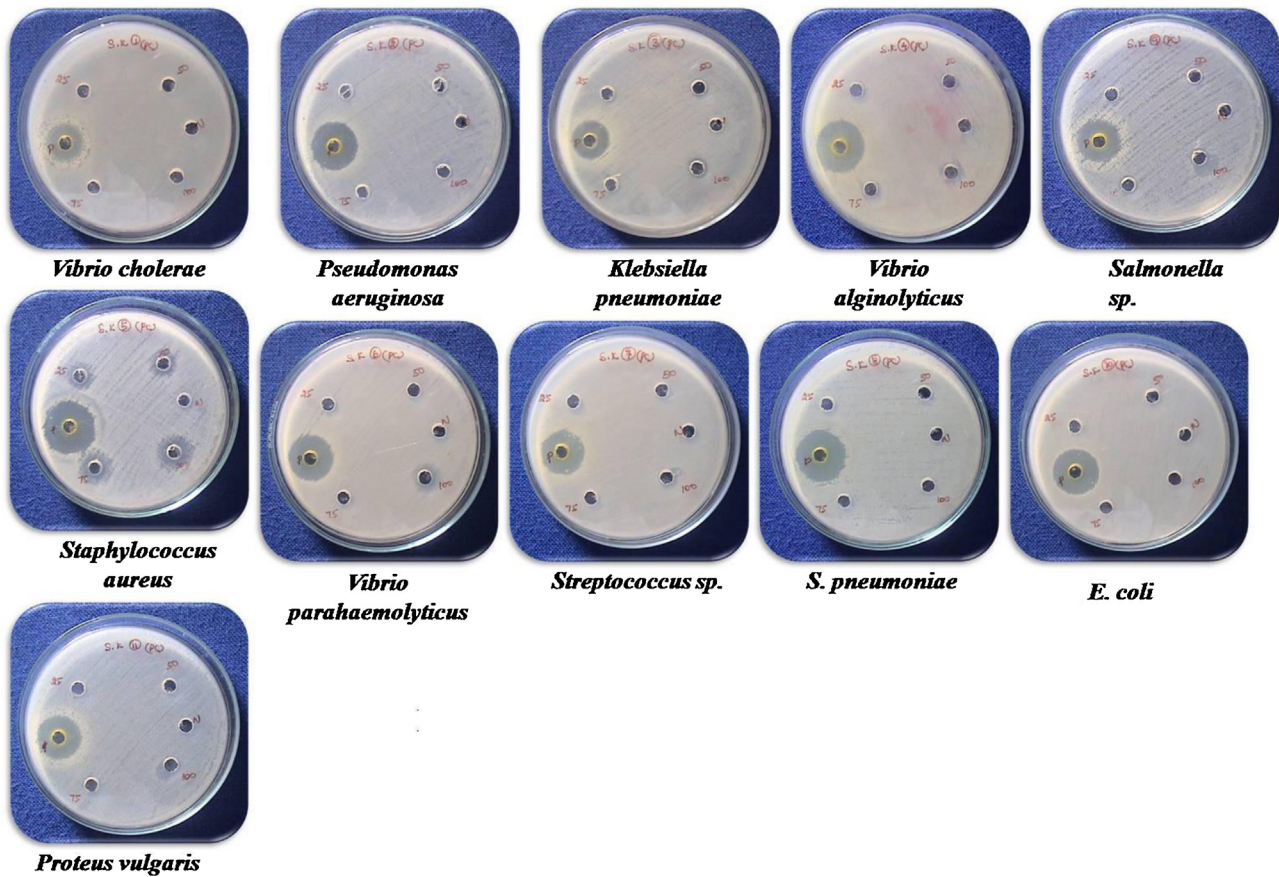


Fig. 3. Antibacterial activity of phosphorylated chitosan from *S. kobsiensis*.

Table 1
Antibacterial activity of chitosan and phosphorylated chitosan from *Sepia kobsiensis*.

S. No	Bacterial strain	Chitosan (%)				Phosphorylated chitosan (%)				+ ^C	- ^C
		25	50	75	100	25	50	75	100		
1	<i>Vibrio cholerae</i>	++	++	++	+++	-	-	+	++	+++	-
2	<i>Pseudomonas aeruginosa</i>	++	++	++	+++	-	-	-	-	+++	-
3	<i>Klebsiella pneumoniae</i>	+	++	++	++	-	-	-	+	++	-
4	<i>V. alginolyticus</i>	+	++	++	++	-	-	-	+	+++	-
5	<i>Staphylococcus aureus</i>	++	++	++	++	+	+	++	+++	+++	-
6	<i>V. parahaemolyticus</i>	++	++	++	++	-	-	++	++	+++	-
7	<i>Streptococcus sp.</i>	+	++	++	+++	-	-	-	-	+++	-
8	<i>S. pneumoniae</i>	+	+	++	++	-	-	-	-	+++	-
9	<i>Salmonella sp.</i>	+	++	++	++	-	-	+	+	+++	-
10	<i>E. coli</i>	+	++	++	++	-	-	-	-	+++	-
11	<i>Proteus vulgaris</i>	++	++	++	+++	-	-	-	+	+++	-

+^C Positive control; -^C Negative control; (-) No activity, (+) Weak activity (7–10 mm dia.), (++) Good activity (11–15 mm dia.), (+++) Very good activity (above 16 mm dia.).

*The statistical significance: *P* values ≤0.05 (DMRT).

maximum 25 mm activity against *P. vulgaris* and minimum 14 mm against *K. pneumoniae*.

3.3. Minimum Inhibitory Concentration (MIC)

MIC values for chitosan against bacterial strains such as *V. cholerae*, *P. aeruginosa*, *K. pneumoniae*, *V. alginolyticus*, *S. aureus*, *V. parahaemolyticus*, *Streptococcus sp.*, *S. pneumoniae*, *Salmonella sp.*, *E. coli* and *P. vulgaris* were recorded as 60, 60, 100, 80, 80, 100, 60, 100, 80, 80 and 50 µg/ml, respectively. Whereas in phosphorylated chitosan, the MIC for *V. cholerae*, *K. pneumoniae*, *V. alginolyticus*, *S. aureus*, *V. parahaemolyticus*, *Salmonella sp.* and *P. vulgaris* was

recorded as 80, 100, 100, 50, 80, 100 and 100 µg/ml, respectively (Table 2).

4. Discussion

The overall goal of the present investigation is not only to compare the ability of antibacterial activity of chitosan and its water soluble phosphorylated chitosan prepared from the cuttlebone of *S. kobsiensis* but also to know whether derivatization of chitosan improves the antibacterial activity. The results of the present investigation clearly showed (Tables 1 & 2) that good antibacterial activity was exhibited by chitosan than the

Table 2
MIC of chitosan and phosphorylated chitosan from *Sepia kobsiensis*.

Bacterial strains	Chitosan ($\mu\text{g/ml}$)								Phosphorylated chitosan ($\mu\text{g/ml}$)							
	100	80	60	50	40	20	10	5	100	80	60	50	40	20	10	5
<i>Vibrio cholerae</i>	–	–	*	+	++	+++	+++	+++	–	*	+	++	++	+++	+++	+++
<i>Pseudomonas aeruginosa</i>	–	–	*	+	++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
<i>Klebsiella pneumoniae</i>	*	+	++	+++	+++	+++	+++	+++	*	+	++	++	+++	+++	+++	+++
<i>V. alginolyticus</i>	–	*	+	++	+++	+++	+++	+++	*	+	++	++	+++	+++	+++	+++
<i>Staphylococcus aureus</i>	–	*	+	++	+++	+++	+++	+++	–	–	–	*	+	++	+++	+++
<i>V. parahaemolyticus</i>	*	+	+	++	+++	+++	+++	+++	–	*	+	++	++	+++	+++	+++
<i>Streptococcus sp.</i>	–	–	*	+	+	++	+++	+++	+	++	++	+++	+++	+++	+++	+++
<i>S. pneumoniae</i>	*	+	++	+++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
<i>Salmonella sp.</i>	–	*	+	+	++	+++	+++	+++	*	+	++	++	+++	+++	+++	+++
<i>E. coli</i>	–	*	+	+	++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
<i>Proteus vulgaris</i>	–	–	–	*	+	++	+++	+++	*	+	++	++	+++	+++	+++	+++

*The statistical significance: P values ≤ 0.05 (DMRT).

phosphorylated chitosan against all the clinically isolated human pathogens and the derivatization did not improve the activity. But at the same time the activity was found to be different with different bacterial strains.

In recent years, great attention has been paid to study the bioactivity of natural products due to their potential pharmacological utilization. The rationale of searching for drugs from marine environment from the fact that marine plants and animals have adapted to all sorts of habitats in the marine environment and these are constantly under tremendous selection pressure including competition for space, predation, surface fouling and reproduction. Many of these organisms are showing antimicrobial properties. Although most of the antibacterial agents isolated from marine sources have not been active enough to compete with classical anti-microbials obtained from microorganisms [12–14], majority of marine organisms are yet to be screened for discovering useful antibiotics.

The FT-IR spectrum of chitosan from the gladius of *S. lessoniana* recorded the peak at 3409.07 cm^{-1} corresponds to H-bonded NH_2 & OH stretching. The peaks at 2921.24 cm^{-1} and 2852.70 cm^{-1} correspond to aliphatic CH stretching. The peak obtained at 1654.79 cm^{-1} corresponds to the amide stretching of C=O and the bands at 1106.85 and 1020.63 cm^{-1} attributed to the C–O–C stretching vibration mode. In phosphorylated chitosan the peak found at 1381.44 corresponds to P=O stretching. The peaks found at 1084.28 and 561.86 are due to P–OH group [12]. In the present investigation, the FT-IR spectrum of chitosan from *S. kobsiensis* also reported the peaks more or less at the same wave length, as in *S. lessoniana* i.e., the peaks corresponding to H-bonded NH_2 and OH stretching at 3435.37 cm^{-1} , aliphatic CH stretching at 2921.94 cm^{-1} and 2852.95 , amide stretching of C=O at 1640.62 cm^{-1} and C–O–C stretching vibration modes at 1020.99 cm^{-1} . In phosphorylated chitosan the peak found at 1386.30 cm^{-1} attributes to P=O stretching, as also noticed (1380 cm^{-1}) in the phosphorylated chitosan in *S. lessoniana* [12].

In an earlier study, 89% of deacetylated chitosan and its oligosaccharides showed more effective activity against pathogens than that of non-pathogens except in the case of lactic acid bacteria [15]. Further, chitosan from *S. lessoniana* reported the maximum inhibition of 14 mm and against *S. aureus* at the highest concentration of 5 mg/ml and minimum inhibition of 8 mm against *K. pneumoniae* and *V. cholera* [8]. But in the present investigation in 100% concentration, the maximum inhibition zone (17 mm diameter clear zone) was noticed against *P. vulgaris* in chitosan, 16 mm clear zone against *S. aureus* in phosphorylated chitosan. The lowest inhibition zone of 13 mm was observed against *S. pneumoniae* in chitosan and 7 mm against *P. vulgaris* in the case of phosphorylated chitosan. The antibacterial activity of chitosan against all pathogenic strains was found concentration

dependent. This result is consistent with the work of Jeon et al. [16] who have also reported the increased antibacterial activity with increase in the concentration of chitosan.

Generally, the inhibitory activity of chitosan towards bacteria is considered in terms of its chemical and structural properties. As a polymeric macromolecule, chitosan is unable to pass the outer membrane of bacteria (since this membrane functions as an efficient outer permeability barrier against macromolecules). Therefore, direct access to intracellular parts of the cell by chitosan is unlikely. A key feature of the chitosan is its positive charge of the amino group at C-2 below its pK_a (pH, 6.3). This creates a polycationic structure, which can be expected to interact with the predominantly anionic components (lipopolysaccharides and proteins) of the bacterial surface [17]. However, it was shown that the biological activity of chitosan significantly depends on its physico-chemical properties such as molecular weight and molecular fraction of glucosamine units in the polymer chain (i.e., the degree of chitosan *N*-deacetylation), pH of chitosan solution and of course, the target microorganism [18,8].

Phosphorylated chitosan from *S. lessoniana* recorded the maximum inhibition of 13 mm against *V. cholerae* and *S. pneumoniae* at the concentration of 5 mg/ml and minimum inhibition of 7 mm against *V. cholera*, *S. aureus*, *S. pneumoniae* and *P. vulgaris* at the concentration of 1.25 mg/ml [12]. When compared to this finding, the present investigation showed a higher antibacterial activity in 100% concentration with the maximum inhibition zone of 16 mm clear zone recorded against *S. aureus* in phosphorylated chitosan and lowest inhibition zone of 7 mm against *P. vulgaris*.

The exact mechanism of antimicrobial action of chitosan derivatives is still unknown, but different mechanisms have been proposed. The water-soluble chitosan derivative is said to be increasing the permeability of cell membrane and ultimately disrupted bacterial cell membrane with the release of cellular contents [19]. The water-insoluble chitosan molecules also precipitate and stack on the microbial cell surface, thereby forming an impervious layer around the cell and blocking the channels, which are crucial for living cells. Such a layer is expected to prevent the transport of essential solutes and may also destabilize the cell wall beyond repair thereby causing severe leakage of cell constituents and ultimately cell death [20,8] which would be the possible reason for the antibacterial activity of chitosan and phosphorylated chitosan from the internal bone of *S. kobsiensis*.

Estimation of Minimum Inhibitory Concentration (MIC) is widely used in the comparative testing of new drugs. In clinical laboratories it is used to establish the susceptibility of organisms that is required for clinical management [21]. In the present investigation, the MIC values for chitosan and phosphorylated chitosan from *S. kobsiensis* against bacterial strains such as *V. cholerae*, *P. aeruginosa*, *K. pneumoniae*, *V. alginolyticus*, *S. aureus*, *V.*

parahaemolyticus, *Streptococcus* sp., *S. pneumoniae*, *Salmonella* sp., *E. coli* & *P. vulgaris* and *V. cholerae*, *K. pneumoniae*, *V. alginolyticus*, *S. aureus*, *V. parahaemolyticus*, *Salmonella* sp. & *P. vulgaris* were reported as 60, 60, 100, 80, 80, 100, 60, 100, 80, 80 & 50 µg/ml and 80, 100, 100, 50, 80, 100 & 100 µg/ml, respectively.

In conclusion, the present investigation reveals that the chitosan from *S. kobeensis* inhibits the growth of all the tested pathogenic bacterial strains when compared to the phosphorylated chitosan which indicates that the chitosan may contain a broad range of antibacterial activity. Additionally the present study brings out the possibility of using the internal bone of cuttlefish that is thrown as waste at home and seafood processing industries are very good and promising source for antibacterials. However, further investigation involving clinical experiments is needed for its use as a drug for human beings.

Acknowledgements

Authors are thankful to the Director and Dean, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, and Annamalai University for providing necessary facilities. One of authors (AS) is thankful to the Centre for Marine Living Resources and Ecology (CMLRE), Ministry of Earth Sciences, Kochi for providing the financial assistance.

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