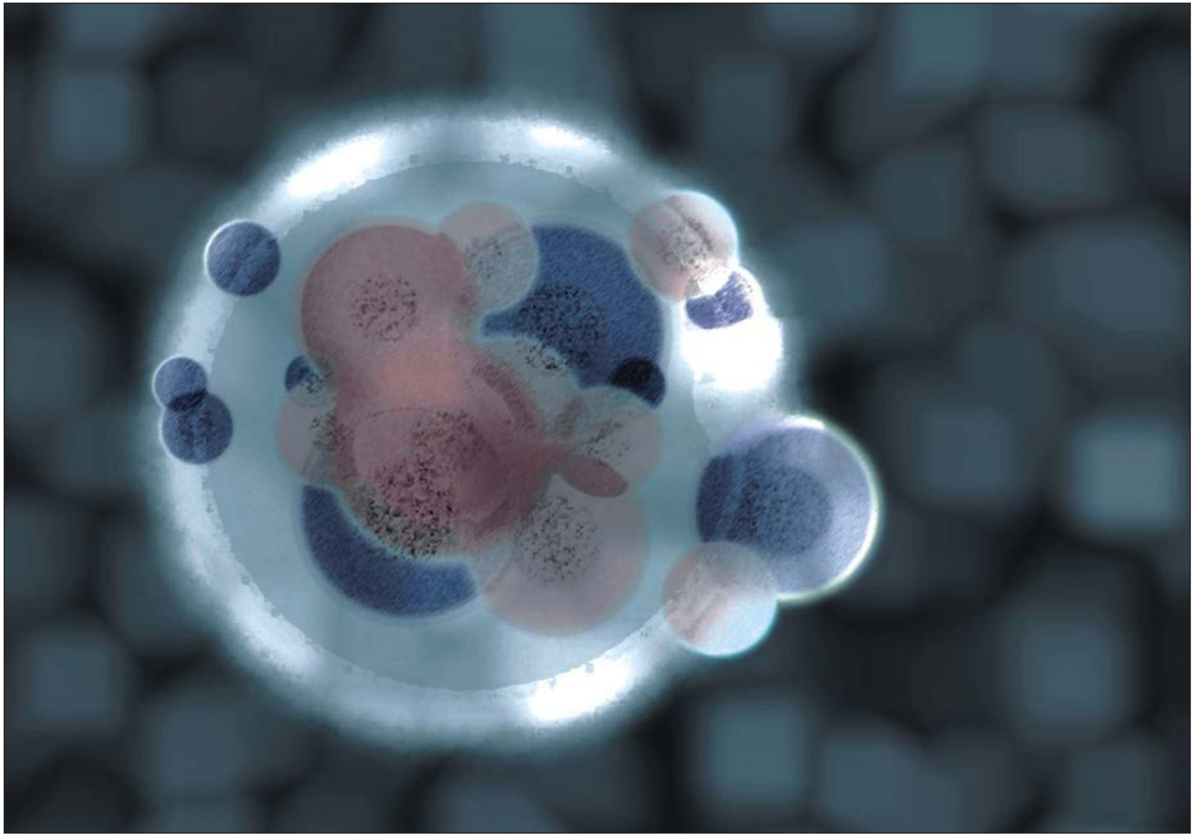

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Preparation of Chitosan Derivatives from Gladius of Squid *Sepioteuthis lessoniana* (Lesson, 1830) and Antimicrobial Potential against Human Pathogens

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Abstract: In this study, the antimicrobial potential of chitosan and its water soluble derivatives from gladius of squid *Sepioteuthis lessoniana* against eleven human pathogens (gram +ve and gram -ve bacteria) was investigated. Chitosan was extracted from the gladius through demineralization, deproteinization and deacetylation. Chitosan was chemically modified by reacting with DMF-chlorosulfonic acid and orthophosphoric acid to yield water soluble derivatives such as sulfated chitosan and phosphorylated chitosan, respectively. The antibacterial activity was assessed by well diffusion technique. Different concentrations of chitosan and water soluble derivatives were analyzed by MIC techniques. The structure of chitosan, sulfated chitosan and phosphorylated chitosan was elucidated by FT-IR spectroscopy. Chitosan showed maximum inhibition of 14 mm against *S. aureus* and minimum inhibition of 8 mm against *K. pnemoniae* and *V. cholerae*. SCL showed maximum inhibition of 14 mm against *V. cholerae* and *E. coli*. PCL showed maximum inhibition of 13 mm against *V. cholerae* and *S. pnemoniae*. Chitosan and its derivatives possess potent antibacterial activity against several pathogens among the eleven pathogens tested and could be used as an alternative antibacterial agent.

Key words: Chitosan, sulfated chitosan, phosphorylated chitosan, *Sepioteuthis lessoniana*

INTRODUCTION

Chitosan is a non-toxic biopolymer derived from the second most abundant polymer chitin by deacetylation. It is found in the exoskeleton of crustaceans, internal shell of cephalopods, cuticles of insects and the fungal cell walls. Chitin in nature should be classified into three forms α, β and γ-chitin based on the raw material (Yen *et al*., 2007). Chitosan have wide range of application in various fields such as medicine, food, pharmaceutical, nutraceutical and agriculture (Li *et al*., 2007). Chitosan could be used as a food preservative (Roller and Covill, 2000) and also used as antimicrobial packaging of films (Yang and Lin, 2002).

Chitosan has a wide inhibition not only against Gram-positive and Gram-negative bacteria but also yeast and moulds. Chitosan has poor solubility which limited its applications as a drug in various fields. The insolubility of chitosan should be overcome by chemical modification that can enhance the biological activity. Water soluble chitosan derivatives were prepared by sulfation and phosphorylation of chitosan. Liu *et al*. (2001) found chitosan and its derivatives possess potent antibacterial activity. According to the structure-activity relationship, multiple derivation of chitosan is quite significant in view of preparing polysaccharide-based advanced materials with multiple functions.

Many investigators reported that antimicrobial effect of chitosan depend on its Molecular weight (Mw) and Degree of Deacetylation (DDA) (Liu *et al*., 2006). Mw and DDA persuade chitosan solubility and consequently interaction with the cell walls of target microorganisms also. Therefore, antimicrobial properties of chitosan derivatives are different. The antimicrobial properties of chitosan obtained from crustacean shells and fungal cell wall have been well studied, only a very few reports on antimicrobial properties of chitosan from cephalopods (gladius and cuttlebone).

The goal of this study was to evaluate the antibacterial properties of chitosan and its water soluble derivatives extracted from the gladius of squid, *S. lessoniana*. In this investigation, water soluble chitosan derivatives were prepared by sulfation and phosphorylation using chlorosulfonic acid and ortho phosphoric acid, respectively. Further, the effect of various concentrations of squid chitosan (β-chitosan)

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and its derivatives on viability of eleven human pathogenic microorganisms was studied and their Minimum Inhibitory Concentrations (MIC) were also measured.

MATERIALS AND METHODS

Sampling and identification: The specimen (*S. lessoniana*) were collected from Cuddalore landing centre (Lat. 11°42' N; Long. 79°46'E), Southeast coast of India. The publication of Roper *et al*. (1984) and Shanmugam *et al*. (2002) were used for identifying the squid.

Extraction and preparation of chitosan and its derivatives: Chitin was extracted from the pulverized internal shell of *S. lessoniana* by demineralization and deproteinization and then converted into chitosan through deacetylation process using 40% NaOH (Takiguchi, 1991a, b) and designated as CL. Fifty milliliter of DMF.SO_3 was added into a 50 mL of chitosan solution in a mixture of DMF-formic acid with swirling to get gelatinous chitosan. Then the reaction was run at adequate temperature (40-60°C) for 1-2.5 h and 95% of ethanol (300 mL) was added to precipitate the product. The mixture of products was filtered through a Buchner funnel under reduced pressure. The precipitate was washed with ethanol and then re-dissolved in distilled water. The pH was adjusted to 7-8 with 2 M NaOH. The solution was dialyzed against distilled water for 48 h using a 12000 Da MW cut-off dialysis membrane. The product was then concentrated and lyophilized (Lark Pneguin (4 kg) Classic Plus)) to give sulfated chitosan (SCL) (Xing *et al*., 2005). Phosphorylated chitosan was prepared by dissolving 2 g of chitosan powder with 30 g of urea and 50 mL of DMF. Then 5.2 mL of orthophosphoric acid was added to the chitosan solution. The mixture was reacted 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. The solution was dialyzed against distilled water for 48 h using a 12000 Da MW cut-off dialysis membrane. Then the product was lyophilized to get phosphorylated chitosan (PCL).

FT-IR spectral analysis: The FT-IR spectral analysis of solid sample of chitosan, sulfated chitosan and phosphorylated chitosan from *S. lessoniana* were relied on an AVATAR 330 Spectrometer. Sample (10 μg) was mixed with 100 μg of dried potassium bromide (KBr) and compressed to prepare salt discs (10 mm diameter) for reading the spectrum.

Determination of antibacterial activity by well diffusion method: Eleven species of bacteria were used as test organisms (Bacterial strains-Gram-positive: *Streptococcus* sp., *S. pneumoniae* and *Staphylococcus aureus*; Gram-negative: *Escherichia coli*, *Vibrio cholerae*, *V. alginolyticus*, *V. parahaemolyticus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* sp. and *Proteus vulgaris*). All the bacterial strains were clinical isolates, obtained from the Raja Muthaiah Medical College Hospital, Annamalai University, Annamalai Nagar, India. Nutrient broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. All the eleven bacterial strains were individually inoculated in the sterilized nutrient broth and incubated at 37°C for 24 h. Mueller Hinton Agar (MHA, Himedia) 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. Stationary phase bacterial cultures were inoculated in the petridishes by using a sterile cotton swab. The antibacterial activity was tested against eleven pathogenic human isolates by Agar well diffusion method (Tepe *et al*., 2004). Twenty four hour 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 and four different concentrations (1.25-5 mg mL^{-1}) of CL, SCL and PCL were prepared by dissolving in appropriate solvent and loaded in the wells. Standard (Tetracycline, 1 mg mL^{-1}) and Control (0.2% acetic acid and/or distilled water) were added into the, respectively labelled wells. The plates were incubated at 37°C for 24 h in upright position. The experiment was carried in triplicates and the zone of inhibition was recorded.

Determination of the minimum inhibitory concentration (MIC): The MIC of chitosan and its derivatives (sulfated and phosphorylated chitosan) was determined by turbidimetric method (Rajendran and Ramakrishnan, 2009). In this method, a stock solution of 100 μg mL^{-1} was prepared. This was serially diluted to obtain various ranges of concentrations between 5 and 100 μg mL^{-1}. To 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 10^5 cells mL^{-1} was then introduced. A set of test 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 its derivatives that inhibited the growth of bacteria was considered as the minimum inhibitory concentration.

Statistical analysis: Data on the inhibitory effects of chitosan, sulfated chitosan and phosphorylated chitosan were analyzed by one-way analysis of variance (ANOVA) using SPSS-16 version software followed by Duncun's Multiple Range Test (DMRT) and standard errors(±). The values at $p<0.05$ were considered for describing the significant levels.

RESULTS

The FT-IR spectrum of CL from gladius of *S. lessoniana* recorded 12 peaks between 462.31 and 3409.55 cm^{-1}; whereas the SCL and PCL showed 14 and 15 peaks lying between 464.00 and 3373.21 cm^{-1} and 614.29 and 3534.25 cm^{-1}, respectively (Fig. 1).

Antibacterial activity of chitosan and its derivatives from *S. lessoniana* against Gram-positive and Gram-negative bacteria was explored by well diffusion method. The capability of chitosan and its water soluble derivatives to inhibit the growth of the tested bacteria on solid media is shown in Table 1. The MIC of the chitosan, phosphorylated chitosan and sulfated chitosan against various microorganisms is shown in Table 2. CL showed maximum inhibition of 14 mm against *S. aureus* at the

Table 1: Antibacterial activities of chitosan, sulfated chitosan and phosphorylated chitosan from *S. lessoniana*

	Chitosan						Sulfated chitosan						Phosphorylated chitosan					
Bacterial strains	P	25%	50%	75%	100%	N	P	25%	50%	75%	100%	N	P	25%	50%	75%	100%	N
Vibrio cholerae	24	8	9	10	11	-	21	7	8	11	14	-	19	7	8	9	13	-
P. aeruginosa	21	-	8	9	10	-	23	-	-	-	9	-	20	-	-	-	-	-
K. pneumoniae	26	8	9	10	11	-	15	-	-	-	-	-	20	8	10	11	12	-
V. alginolyticus	25	-	8	10	11	-	20	-	-	-	-	-	20	-	-	-	-	-
S. aureus	16	9	10	12	14	-	21	-	-	8	10	-	20	7	8	10	12	-
V. parahaemolyticus	28	10	11	12	13	-	22	-	-	-	12	-	21	-	-	-	-	-
Streptococcus sp.	26	9	10	11	12	-	20	-	-	7	9	-	16	-	-	-	-	-
S. pneumoniae	26	-	-	-	-	-	24	-	-	-	-	-	24	7	9	11	13	-
Salmonella sp.	25	-	-	-	10	-	20	-	-	-	-	-	18	-	-	-	-	-
E. coli	26	9	10	12	14	-	21	7	9	11	14	-	15	-	-	-	-	-
Proteus vulgaris	26	-	-	-	-	-	18	-	-	-	-	-	20	7	8	9	11	-

Positive control: Tetracycline (1 mg mL^{-1}), 25-100%: Samples at the range of 1.25-5 mg mL^{-1}, Negative control: Either acetic acid or distilled water

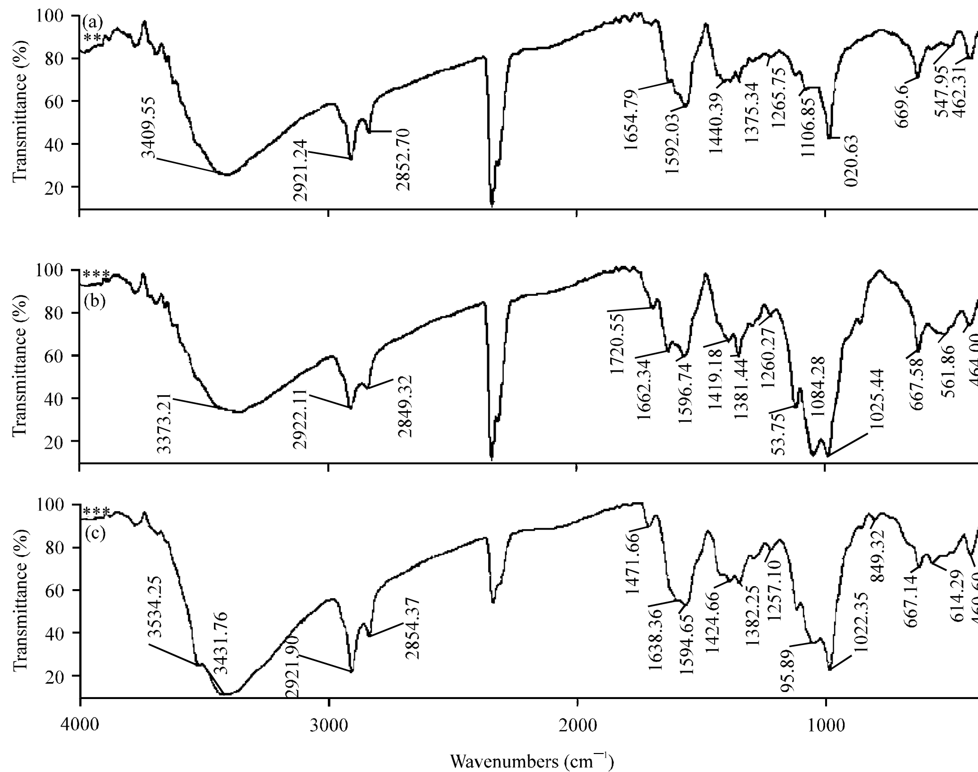

Fig. 1(a-c): FT-IR spectral analysis of (a) Chitosan, (b) Phosphorylated chitosan and (c) Sulfated chitosan from *S. lessoniana*

Table 2: MIC of chitosan, sulfated chitosan and phosphorylated chitosan against tested microorganisms

	Chitosan (μg mL^{-1})								Sulfated chitosan (μg mL^{-1})								Phosphorylated chitosan (μg mL^{-1})							
Bacterial strains	100	80	60	50	40	20	10	5	100	80	60	50	40	20	10	5	100	80	60	50	40	20	10	5
Vibrio cholerae	-	*	+	++	+++	+++	+++	+++	-	-	*	+	++	+++	+++	+++	-	*	+	++	+++	+++	+++	+++
P. aeruginosa	*	+	++	++	+++	+++	+++	+++	*	++	++	++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
K. pneumoniae	*	+	++	++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++	-	*	+	++	+++	+++	+++	+++
V. alginolyticus	-	*	+	++	+++	+++	+++	+++	+	++	++	++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
S. aureus	-	-	*	+	++	+++	+++	+++	-	*	+	++	+++	+++	+++	+++	-	*	+	++	+++	+++	+++	+++
V. parahaemolyticus	-	-	*	+	++	+++	+++	+++	-	*	+	++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
Streptococcus sp.	-	*	+	++	++	+++	+++	+++	*	+	++	++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
S. pneumoniae	+	++	++	+++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++	-	*	+	++	+++	+++	+++	+++
Salmonella sp.	*	+	++	++	+++	+++	+++	+++	++	++	++	+++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
E. coli	-	-	*	+	++	+++	+++	+++	-	-	*	+	++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++
Proteus vulgaris	+	++	++	+++	+++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+++	*	+	++	++	+++	+++	+++	+++

*: MIC concentration, -: No growth, +: Cloudy solution, ++: Turbid solution and +++: Highly turbid solution

highest concentration of 5 mg mL^{-1}. CL showed minimum inhibition of 8 mm (at the concentration of 1.25 mg mL^{-1}) against *K. pnemoniae* and *V. cholerae*. In the present study, the SCL showed maximum inhibition of 14 mm against *V. cholerae* and *E. coli* at the concentration of 5 mg mL^{-1}. SCL showed minimum inhibition of 7 mm against *V. cholerae* and *E. coli* at the concentration of 1.25 mg mL^{-1}. PCL showed maximum inhibition of 13 mm against *V. cholerae* and *S. pnemoniae* at the concentration of 5 mg mL^{-1}. PCL showed minimum inhibition of 7 mm against *V. cholerae*, *S. aureus*, *S. pnemoniae* and *P. vulgaris* at the concentration of 1.25 mg mL^{-1}.

DISCUSSION

In recent years, great attention has been paid to the bioactivity of natural products because of their potential pharmacological utilization. Most homeopathic medicines are either plant or animal origin. Several molecules extracted from marine invertebrates, including bivalves and cephalopods possess broad spectrum antimicrobial activities affecting the growth of bacteria, fungi and yeasts (Ramasamy *et al*., 2011).

The FT-IR spectrum of chitosan from the gladius of *S. lessoniana* shows the peak at 3409.07 cm^{-1} corresponds to H-bonded NH_2 and OH stretching. The peaks at 2921.24 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 vibrations modes. The FT-IR spectrum of sulfated chitosan indicates the band at 1257.10 cm^{-1} which corresponds to the asymmetric stretching vibrations of SO_3. The peak found at 1381.44 cm^{-1} corresponds to P = O stretching. The peaks found at 1084.28 and 561.86 cm^{-1} were due to P-OH group. This result was correlated with other researcher (Jayakumar *et al*., 2008) who has also reported that the broad peak obtained at 3500 cm^{-1} was due to P-OH group. The peak obtained at 1380 cm^{-1} can be attributed to P = O stretching. The peaks found at 1050 and 500 cm^{-1} were due to P-OH group.

Chitosan with 89% deacetylation and its oligosaccharides showed more effectual activity against pathogenic bacteria than that of non-pathogens (Jeon *et al*., 2001). The minimum inhibitory concentration of chitosan against both gram-negative and gram-positive bacteria was lesser than 0.06%. The MIC value of chitosans for *E. coli* and *S. aureus* was 0.025 and 0.5%, respectively (Uchida *et al*., 1989). Recently water soluble chitosan derivatives have been developed and their antimicrobial activity against several bacteria such as *E. coli*, *S. aureus*, *B. subtilis*, *P. aeruginosa* and *S. mutans* were investigated (Avadia *et al*., 2004). The antibacterial activity of chitosan and its water soluble derivatives against all pathogenic strains was concentration dependent. Furthermore, the results showed that the antimicrobial activity of the compounds have relationship with their concentration and higher concentration result in higher antimicrobial activity. The result of present study consistent with the research of (Liu *et al*., 2006) who had demonstrated that with the increase of the concentration, the antibacterial activities of chitosan had enhanced.

The inhibitory activity of chitosan towards bacteria should be considered in terms of its chemical and structural properties. As a polymer, chitosan is unable to cross 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 cells by chitosan is implausible. Chitosan having positive charge at C2 position of the amino group below pKa value of pH 6.3 which forms a polycationic structure that interacts with the anionic components such lipopolysaccharides and proteins of the bacterial surface (Nikaido, 1996). Further, the chitosan derivative is mainly anionic nature at neutral condition, so the adsorption and binding of cationic group are not so effective. The degree of protonation of NH_2 in chitosan is constant when the pH value is given (Chen *et al*., 2007). When the pH values become higher the degree of protonation of NH_2 becomes lower. Since, the antibacterial test was investigated in sterile distilled water, the amino group is free and has strong coordination ability.

Antimicrobial activity of chitosan has been reported against many strains of bacteria, filamentous fungi and yeasts. However, 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 the target microorganism (Tsai and Hwang, 2004; No *et al*., 2006). Antibacterial activity of chitosan derivatives can also be closely related to the formation of hydrophobic micro-area. At pH 7, the degree of protonation of NH_2 is very low, that is, the repulsion of NH_3^+ is weak and so the strong intermolecular and intra-molecular hydrogen bond results in the formation of hydrophobic micro-area in polymer chain (Chen *et al*., 2007). At the same time, the carboxyl group in the polymer chain is strongly hydrophilic. Therefore, the polymer chains have hydrophobic and hydrophilic parts. This amphiphilic structure provides structure affinity between the cell walls of the bacteria and the chitosan derivative.

The exact mechanism of the antimicrobial action of chitosan derivatives is still unknown, but different mechanisms have been proposed. Water-soluble chitosan increased the permeability of cell membrane and ultimately disrupted bacterial cell membranes with the release of cellular contents (Helander *et al*., 2001). The water-insoluble chitosan molecules can 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 can be 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 (Rhoades *et al*., 2006). The possible reasons for the antimicrobial activity of the SCL and PCL may be the increased permeability of cell membrane which ultimately disrupted bacterial cell membranes with the release of cellular contents.

CONCLUSION

Beta-chitosan from *S. lessoniana* and its water soluble derivatives phosphorylated chitosan and sulfated chitosan showed potent antibacterial activity against human pathogenic bacterial strains. The antibacterial mechanism of chitosan is due to the amino group at the C2 position of the glucosamine residue. These results further support the idea that squid gladius which is thrown as waste can be promising sources of potential antimicrobial agents.

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