

## A Study on the Antimicrobial Activities of Chitin and Chitosan Extracted from Freshwater Prawn Shells (*Macrobrachium Nipponense*)

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### Abstract

**Background:** Chitin and its deacetylated derivative, chitosan, are unique biopolymers. Owing to their properties such as biocompatibility, biodegradability, and antimicrobial and antioxidant activities, they have been widely applied in various industries. The aim of the study was to investigate the antimicrobial activities of chitin and chitosan extracted from freshwater prawn shells.

**Methods:** In this research, prawn shell (*Macrobrachium nipponense*) is used as a source of chitin for the extraction of this valuable biopolymer. The inhibition zone of different concentrations (5, 7.5, and 10 mg/mL) of chitin and chitosan was examined for *in vitro* antibacterial activity against seven kinds of bacterial strains and two fungi (*Aspergillus niger* and *Candida albicans*). Furthermore, minimum inhibitory concentration and minimum lethal concentration were determined.

**Results:** Chitosan had a more inhibitory effect than did chitin. Chitosan demonstrated the maximum inhibitory effect in *Vibrio cholerae* Ogawa, whereas the lowest value was observed in *Escherichia coli* ( $P < 0.05$ ). Fungal organisms were revealed to be bacterial pathogens more resistant to the chitin and chitosan that were extracted from prawn shell. Also, chitin and chitosan showed maximum inhibitory effects on *A. niger*. The lowest minimum inhibitory concentration for chitin and chitosan was between 0.005% and 0.01%, and 0.005% and 0.1%, respectively.

**Conclusions:** Chitosan showed greater antibacterial effect than did chitin against studied bacteria particularly *V. Cholerae* Ogawa and *Staphylococcus aureus* and also revealed good antifungal effects. Thus, chitosan may be used as a source of antimicrobial agent for medical and pharmaceutical applications.

**Keywords:** Chitin, Chitosan, Antibacterial, Prawn, Antifungal.

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## Introduction

The primary defensive shield of crustaceans against pathogenic organisms is their hard exterior skeleton.<sup>1,2</sup> The peculiar qualities of crustaceans come from the bioactive compounds including chitin and chitosan.<sup>3</sup> Chitin is a natural polymer with antimicrobial properties and is naturally found on the shell of the crustacean body such as in crabs and shrimps.<sup>4</sup> Chitin, as the second most abundant natural polysaccharide found in nature, is composed of N-acetyl-D-glucosamine.<sup>5,6</sup> Polymeric chitosan, as a biomaterial obtained through different chemical processes within chitin, is more useful and soluble than chitin is. Both chitin and chitosan have high commercial value because of their varying biological activities (figure 1).<sup>7</sup>

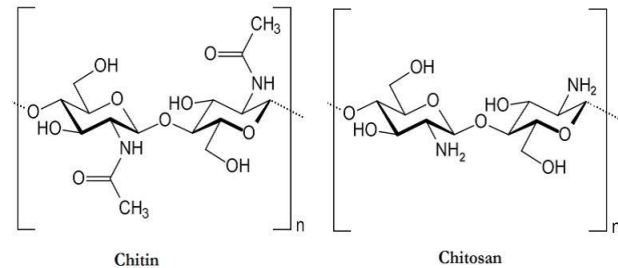


Figure 1. Chemical structure of chitin and chitosan

Chitin and chitosan are used as bioactive compounds with antifungal<sup>7</sup> and bactericidal agents.<sup>8,9</sup> The basic mechanism restraining the microbial activities by chitosan includes the obstruction in the duplication of RNA from DNA through absorption of penetrated chitosan within DNA molecules.<sup>10-12</sup> Under such a mechanism, the molecular weight of chitosan should be lower to penetrate within the cells.<sup>13,14</sup> The electrostatic adhesion of polycationic chitosan to the outer layer of bacterial cells and its destructions is considered as another major inhibitory mechanism function.<sup>15</sup>

The increasing resistance to antibiotics and pathogenic bacteria is currently one of the major challenges that medical centers and hospitals are facing in their treatment efforts.<sup>16,17</sup> Recently, extensive research and studies to search for new microbicide substances derived from marine natural products are being undertaken.<sup>3</sup> The present study focuses on comparing and contrasting antimicrobial effects of chitin and chitosan extracted from freshwater prawn shell against pathogenic bacteria.

## Materials and Methods

Freshwater prawns ( $4.7 \pm 0.12$  cm and  $2.8 \pm 0.1$  g) were caught from the southern Caspian Sea coastal waters using collapsible traps during the summer of 2014. The prawns were first anesthetized using ice-cold shock and then washed, and the shells were removed from their bodies manually. The shells were dried at 55 °C in an oven for 120 min, ground, and then passed through a 25-µm mesh sieve.<sup>18</sup>

The grounded exoskeleton (20 g) was mixed with 200 mL of 7% HCl in 25 °C for 24 h. The sample was filtered and washed with distilled water until it was completely free of acid. The demineralized sample was dried and weighted. Deproteinization was performed using NaOH (0.5 N) for 20 h

in 25 °C, and then the sample was autoclaved for 30 min. Afterward, the sample was washed several times with distilled water and then filtered. The derived chitin was dried in an oven at 100 °C. Deacetylation of chitin to chitosan was carried out using strong NaOH (50%) solution in 60 °C for 2 h. Next, the resulting chitosan was dried and weighted. The deacetylation degree of chitosan was measured on the basis of standard UV spectra (205 nm) using acetyl glucosamine and n-glucosamine hydrochloride.<sup>18</sup> The percentages of these polymers were calculated by dividing the obtained dry weight of chitin and chitosan by the weight of the original grounded shells.

*Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 465), *Bacillus cereus* (PTCC 1154), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (PTCC 1310), and *Vibrio cholerae* (ATCC14035) and two fungal strains *Candida albicans* (PTCC 5027) and *aspergillus niger* (PTCC 5223) provided by the Iranian Research Organization for Science and Technology were used as the tested organisms. Then, bacterial suspensions based on standard 0.5 McFarland ( $1.5 \times 10^8$  CFU/mL) were prepared.<sup>19</sup>

In vitro antibacterial activities of different extracts of prawn shells were evaluated against five microbial stains by using the well-agar diffusion method.<sup>19,20</sup> Stock cultures were added to Muller–Hinton broth on the day before the experiment and incubated for 24 h at 37 °C. Different cultures of pathogenic bacteria were swabbed on the Muller–Hinton agar plates. Furthermore, the filter paper discs (6 mm diameter) were impregnated with exact amounts of each extract. Standard antibiotic disks gentamicin 10 and erythromycin (Iran Daru Company) were used as the positive controls.

The plates were incubated at 37 °C for 24 h. afterward, the inhibition zones that formed on the media were measured.<sup>19</sup> The positive antimicrobial activities were recorded on the basis of the growth inhibition zone. All inhibition assays and controls were carried out in triplicate.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration of chitin and chitosan were measured using serial dilution.<sup>3,18</sup> Chitosan solution (1% w/v in a stoichiometric amount of HCl) was added to Muller–

Hinton broth to get final concentrations of chitosan of 0.1%, 0.075%, 0.05%, 0.025%, 0.01%, and 0.005% (w/v). Each well of the microplates included 50 µL of the chitosan solution, 40 µL of the growth medium, and 10 µL of the inoculum (106 CFU/mL).<sup>3</sup> After incubating the microplates at 37 °C, the p-iodonitrotetrazolium Violet (40 µL in dissolved water) was added to the wells and incubated again at 37 °C for 30 min. Biological active microorganisms after exposure to the p-iodonitrotetrazolium violet produce red color owing to the formulation of formazan. Therefore, the colorlessness of the solution in wells after incubation time indicates the inhibition of bacterial growth.<sup>3</sup>

The data analysis was carried out using SPSS software version 19. The Kolmogorov–Smirnov test was applied for data normalization. In addition, the two-way analysis of variance was applied to compare the antimicrobial property of chitin and chitosan. Finally, Tukey honestly significant difference test was used for cross-sectional comparison of the treatments. The significant level was set at 0.05.

## Results

According to the agar diffusion method, extracted chitin and chitosan from prawn shells inhibited the growth of all gram-negative and gram-positive bacteria (table 1). The antimicrobial efficiency of chitin is lower than that of chitosan. The zone of inhibition measured for chitin ranged between  $7.2 \pm 0.12$  and  $12.4 \pm 0.31$  mm, whereas the values related to chitosan were  $7.1 \pm 0.3$  and  $14.8 \pm 0.41$  mm, which were observed in *V. cholerae* Ogawa and *E. coli*, respectively. The lowest MIC and MLC of both chitin and chitosan were observed in *S. aureus* (0.005% and 0.01%, respectively), and the highest MIC was in *E. coli* (0.1%) wherein no bactericidal effect was observed. The minimum levels of MIC and MLC were exhibited against *V. cholerae* Ogawa and *B. subtilis* in prawn shell chitosan (table 2). The sensitivity of fungal strains to the chitin and chitosan turned out to be lower on bacterial strains (table 3). The results revealed that chitosan had a higher inhibitory antifungal effect on *A. niger* and *C. albicans* as compared with chitin ( $P=0.015$ ).

**Table 1. Mean of inhibitory zone (mm) of extracted chitin and chitosan from prawn shell against bacterial pathogens**

Species	Chitin	Chitosan	Erythromycin	Gentamycin
	200 µg/L	200 µg/L	15 µg/disc	10 µg/disc
<i>S. aureus</i>	$12.4 \pm 0.31^{aA}$	$11.7 \pm 0.3^{cC}$	$7.05 \pm 0.25^{bB}$	$11.6 \pm 0.2^{aC}$
<i>B. Subtilis</i>	$9.8 \pm 0.43^{cB}$	$14.4 \pm 0.11^{bB}$	$14.1 \pm 0.7^{bA}$	$16.8 \pm 0.1^{aB}$
<i>B. cereus</i>	$7.2 \pm 0.12^{cC}$	$8.67 \pm 0.3^{cD}$	$13.6 \pm 0.6^{bA}$	$15.9 \pm 0.1^{aB}$
<i>P. aeruginosa</i>	$12.24 \pm 0.1^{cA}$	$13.92 \pm 0.4^{bB}$	$14.2 \pm 0.21^{bA}$	$18.2 \pm 0.42^{aA}$
<i>E. coli</i>	$7.2 \pm 0.2^{cC}$	$7.1 \pm 0.3^{bA}$	$6.9 \pm 0.2^{bB}$	$8.4 \pm 0.1^{bD}$
<i>K. pneumoniae</i>	$9.33 \pm 0.1^{bB}$	$13.4 \pm 0.12^{aB}$	$7.9 \pm 0.31^{cB}$	$9.8 \pm 0.15^{bD}$
<i>V. cholerae</i> Ogawa	$11.7 \pm 0.31^{bA}$	$14.8 \pm 0.41^{aB}$	$6.25 \pm 0.11^{cB}$	$7.8 \pm 0.1^{cD}$

Different small letters in each row and different capital letters in each column indicates significant differences ( $P < 0.05$ )

**Table 2. The minimum inhibitory concentrations (MIC) and the minimum lethal concentration (MLC) of extracted chitin and chitosan against bacterial and fungal organisms (Concentrations tested. 0.1%, 0.075%, 0.05%, 0.025%, 0.01%, and 0.005%)**

Species	Chitin		Chitosan	
	MIC (%)	MLC (%)	MIC (%)	MLC (%)
<i>S. aureus</i>	0.005	0.01	0.005	0.01
<i>B. Subtilis</i>	0.01	0.025	0.01	0.05
<i>B. cereus</i>	0.01	*	0.01	*
<i>P. aeruginosa</i>	0.01	0.025	0.025	0.05
<i>E. coli</i>	0.05	*	0.1	*
<i>K. pneumoniae</i>	0.025	*	0.025	*
<i>V. cholerae</i> Ogawa	0.025	0.05	0.025	0.075
<i>A. niger</i>	0.025	0.05	0.025	0.05
<i>C. albicans</i>	0.01	*	0.01	0.025

\*Not detected

**Table 3. Mean of inhibitory zone (mm) of extracted chitin and chitosan from prawn shell against fungal strains**

Species	Chitin	Chitosan	Penicillin (30 µg/disc)
<i>A. niger</i>	9.34±0.11 <sup>bb</sup>	10.74±0.1 <sup>bb</sup>	16.2±0.2 <sup>ba</sup>
<i>C. albicans</i>	10.16±0.3 <sup>bb</sup>	12.08±0.1 <sup>bb</sup>	18.4±0.7 <sup>ba</sup>

Different small letters in each row and different capital letters in each column indicate significant differences (P<0.05)

## Discussion

According to our data, chitosan showed the highest growth inhibitory effect on *V. cholerae* Ogawa and had the least effect on *E. coli*. The greater inhibitory effect was observed in chitosan rather than in chitin. In comparison with the usual antibiotic, in all cases, chitosan was better in inhibiting bacterial growth; in other words, bacteria resistant to common antibiotics were sensitive against chitosan. Extracted chitin and chitosan also could minimize the growth rate of the fungus and have shown considerable inhibitory effect on mycelia growth. The antimicrobial impacts of chitin and chitosan along with their derivatives have been reported on many pathogenic agents.<sup>1,13,19,21</sup> The antimicrobial characteristics of chitin and its derivatives have resulted in their widespread application in most of the disinfectant materials.<sup>22,23</sup> The existence of antimicrobial compounds within crustacean shell indicates that such characteristics play a crucial role in the protection of these aquatic animals from pathogenic organisms.<sup>24</sup>

In their works, Sasikala and Chitra<sup>24</sup> investigated the antimicrobial effects of extracted chitosan from *Macrobrachium rosenbergii* shells and found that their effects surpassed those of chitin. It was also noticed in the present study that chitosan derived from prawn shows a greater antimicrobial impact. Chitosan extracted from crab exoskeleton indicated the highest inhibitory effect against *S. aureus*.<sup>25</sup> Chitosan extracted from the shells of black tiger shrimp<sup>26</sup> revealed the highest antimicrobial effect on *E. coli* (MIC=625 ppm), which is contrary to the results obtained in this study. The antibacterial effect is a complicated stage, which, owing to cell surface characterization, differs between gram-positive and gram-negative bacteria. Chitosan has been reported to have a stronger bactericidal effect on gram-positive bacteria than on gram-negative bacteria. This may be because of the outer membrane of the gram-negative bacteria,<sup>27</sup> although in the present study, the *V. cholerae* Ogawa, which showed better inhibitory effect, is a gram-negative bacteria.

The functional mechanism of chitin and chitosan as antimicrobial agents is not yet fully understood. However, it is assumed that the interaction between positive charges on the surface of chitin and chitosan molecules with the negative charges on the membrane of the pathogenic cells could be a possible mechanism.<sup>28</sup> According to the Diaz-Rojay et al. study, the high polycationic property of chitosan rather than chitin might show greater antimicrobial activity in this polymer. In fact, the higher antibacterial qualities of chitosan might be attributed to their greater proton donor capacity of amino groups, which can react with negative charges of a molecule's surface and result in the destruction of bacterial cells.<sup>29</sup>

The inhibitory function of chitosan on fungal growth was shown to be higher than that of chitin in some studies.<sup>28</sup> Type of fungal species and molecular weight are considered as effective factors in the level of antifungal activity.<sup>3</sup> Li et al. mentioned that antifungal activity increases with decrease of chitosan molecular weights.<sup>30</sup> However, the most important antifungal inhibitory of chitin and chitosan can depend on the type of fungus.<sup>3</sup> Possible mechanisms for antifungal activity of this biopolymer, which have been proposed, are as follows: 1) increasing permeability of membrane due to interaction of chitosan with negatively charged fungi membrane, 2) adverse effect on the production of essential proteins and enzymes due to changes in the DNA structure, and 3) unavailability of essential nutrients for fungal growth through chelation by chitosan. So, differences in the antifungal activity depend on the type of inhibitory mechanism of chitosan, which can vary in different fungal species.<sup>3,31</sup>

The findings of this study suggest that the greater antibacterial effect of chitin and chitosan obtained from prawn shells surpasses their antifungal properties.<sup>32</sup> The previous investigations concerning the microbicide effects of chitin and chitosan also indicated that both biopolymers revealed more antibacterial activities than antifungal effects.<sup>32</sup> A milder antifungal effect might be accounted for by the lower impacts of antimicrobial compounds present on fungal cell walls composed mainly of chitin and glucagon.<sup>33</sup> However, in a research carried out by Burrows et al., it was detected that the antifungal effects related to crab shell are far superior to the antibacterial impacts.<sup>34</sup>

The difference between the results of this study and those of previous researches conducted on antimicrobial effect of these biopolymers could be related to various factors such as type of chitosan, microorganism, deacetylation degree, solvent, and the pH, which should be considered in the study of antimicrobial properties of chitin and chitosan.

In summary, it can be concluded that extracted chitosan from prawn shell exhibited greater bacteriostatic effect and antifungal activity than did chitin. But further study that pays more attention on the mode of action of chitin and chitosan is needed.

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## Conflict of Interest

The authors declared that they have no conflict of interest.

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