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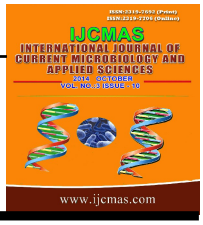


**Original Research Article**

# Action of Chitosan and its derivatives on clinical pathogens

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**Keywords**

Chitosan; Glucosamine; Antibacterial activity; *Staphylococcus aureus*; Coagulase negative *Staphylococcus aureus*; *Enterococcus*.

**A B S T R A C T**

Chitosan and its derivatives which are known to possess multiple functional properties , have attracted considerable interest due to their biological activities and potential applications in the pharmaceutical, food, agricultural and environmental industries. Many researchers have focused on chitosan as a potential source of bioactive materials in the past few decades. This study focuses on the antibacterial activity of chitosan and its derivatives against clinical pathogens and comparing the antibiotic sensitivity pattern of chitosan using standard antibiotic discs. The inhibitory activity of chitosan was greater against *Staphylococcus aureus* than Coagulase negative *staphylococcus* and *Enterococci*. The antibacterial activity of chitosan against ATCC strain of *Staphylococcus aureus* (ATCC 25923) at varying concentrations (100 - 400µg) was greater than standard antibiotics like Vancomycin and Amoxyclav commonly used against these pathogens. The Minimum Inhibitory Concentration of chitosan (1%) for *Staphylococcus aureus* (ATCC 25923) was 100µg. Chitosan hydrolysates obtained from both chemical ($H_2O_2$ in the presence of ferric ions) and enzymatic (lysozyme) hydrolysis were not as inhibitory as the native one, except that from chemical hydrolysis with zinc. Glucosamine showed no visible activity against any of the organism tested. The bioactivity summarized here may provide novel insights into the functions of chitosan and potentially enable its application as a safe antimicrobial agent against clinical pathogens.

## Introduction

With the emergence of potent antibiotic resistant pathogenic microorganisms, there is increasing demand for more natural and safer antimicrobial agents. In that regard, much attention has been focused on the safety and efficiency of chitosan from animal origin as a natural antimicrobial. Chitosan has several advantages over other types of natural antimicrobials, such as higher antimicrobial activity, broader spectrum of activity, higher killing rate and lower toxicity towards mammalian cells.

Chitosan is a natural non- toxic biopolymer produced by the de-acetylation of chitin, a major component of the shells of crustaceans. It is also found in various insects, worms, fungi and mushrooms in varying proportions from species to species and from region to region. Chemically chitosan is a poly –β (1,4) – glucosamine (*Knorr*,1984). *Chen et al* (2003) applied chitosan as a natural disinfectant against waterborne pathogens and proved it to be promising.

Apart from microbial strains, antimicrobial activity of chitosan varies widely, depending on % DD, molecular weight(MW), pH, temperature and the presence of interfering substances such as proteins, fats and other antimicrobials (*Rhoades* and *Roller*, 2000; *Tsai et al*, 2000; *Jeon et al*, 2001; *Knowles* and *Roller*, 2001; *No et al*, 2002; *Zheng* and *Zhu*, 2003). Furthermore, synergistic effects of chitosan covalently bonded with lysozyme have been reported by *Song* and others (2001). Inherent antimicrobial properties of chitosan and its high-film forming and entrapping ability could be the primary driving force in the development of new applications for this underused biopolymer. Like lysozyme, chitosan may be combined with other active antimicrobial substances for enhancing its antimicrobial efficacy (*S.I.Park, et al*.,2004).

The reactive functional group present in chitosan (amino group at the C2 position of each de acetylated unit and hydroxyl groups at the C6 and C3 positions) can be readily subjected to chemical derivatization allowing the manipulation of mechanical and solubility properties enlarging its biocompatibility( *Goy.R.C et al*.,2009).

*In vivo* experiments using a mouse model of *Staph*. *aureus* infected wounds confirmed the effectiveness of chitosan acetate bandage improving wound healing (*Burkatovskaya et al*, 2008). Chemical modification by acetylation, results in increased water solubility (*Chi et al* 2006).

In this context, chitosan and its derivatives has been explored for its antimicrobial activity against clinical pathogens. Even though studies on antimicrobial and antifungal activity of chitosan for food preservation are being done extensively, such reported investigation is considerably less for clinical applications.

*Staph. aureus*, Coagulase negative *Staph*. *aureus* and *Enterococci* are common pathogens responsible for healthcare associated infections as well as community acquired ones. They were the etiological factors of a wide spectrum of infections. The therapeutic problems are caused by resistance of *Staph*. *aureus* to many antibiotics, specifically to methicillin (methicillin resistant *Staph*. *aureus*), in such cases a limited spectrum of antibiotics may be used and prolonged hospitalization is expensive. Hence there is an urgent need for the development of alternative antimicrobial therapeutics.

## Materials and Methods

### Materials

Chitosan and glucosamine were obtained as a gift from Central Institute of Fisheries Technology (CIFT), Kochi, Kerala. Different clinical isolates of *Staphylococcus aureus*, Coagulase negative *Staphylococcus* and *Enterococcus spp* were procured from clinical lab. Media and antibiotic discs – Muller Hinton Agar (MHA), Nutrient Agar (NA), Peptone Water (PW) were obtained from HIMEDIA, Mumbai. All microbiological media, reagents and enzymes used were obtained from Spectrum

Chemicals (P) ltd, USA, Qualigens Company ltd, Mumbai and Loba Chemi Company ltd, Mumbai.

## Methods

### Preparation of Chitosan

The chitosan obtained was dissolved in 1% acetic acid, with degree of de-acetylation of 90% and 82%, and viscosity measured were 90 cP and 270 cP respectively using Fungi lab Visco Basic Plus. The molecular weight for 90+ DD chitosan is 7.216 $x10^3$ Daltons.

### Clinical Isolates and their Cultivation

Clinical isolates of *Staph. aureus*, Coagulase negative *Staph. aureus* and *Enterococci spp* were stored in Nutrient Broth (NB) and sub-cultured on Nutrient Agar (NA) plates and incubated at $37^0$C overnight. ATCC strain of *Staph. aureus* (ATCC 25923) was used as standard organism for the study.

### Preparation of Chitosan derivatives

### Oxidative-reductive degradation with $H_2O_2$

About 30 mg of 82% DD chitosan was dissolved in 3ml acetic acid (1%). Aliquots of the chitosan solution were mixed with 1ml of aq: $FeCl_3$ (10mM). $H_2O_2$ (1M) is added to final concentrations of 0.2, 1, 5, 10, 25 and 50mM and the volume of each reaction mixture was made up to 5mL by adding sterile distilled water. Degraded chitosan solutions were stored at $4^0$C for not more than 24 hrs before antimicrobial activity was tested. The native and degraded chitosan solutions that was autoclaved at $121^0$C for 15 min were used to test the antibacterial activity against *Staphylococcus aureus*, Coagulase negative *staphylococcus* (CoNS) and *Enterococci spp*.

***Hydrolysis with Lysozyme***: Lysozyme having an activity of 50,000U/mg of protein was dissolved in 0.1 M KCl (1mL) and added to a solution containing 30 mg of chitosan in 10mM acetic acid – sodium acetate buffer (pH: 4.5), so that the final enzyme concentration is 0.025% (wt/v).The reaction mixture is stirred at room temperature. The samples were autoclaved at $121^0$C for 15 min to inactivate the enzyme and to sterilize. Then it is used for testing the antimicrobial activity *in vitro*.

### Antibacterial activity determination using glucosamine

Glucosamine (12mg) was dissolved in 1ml of acetic acid (30%).The pH value was adjusted to 7.0 by adding 0.1M NaOH. Antibiotic sensitivity was performed by using this solution. Acetic acid (1%, pH.6) was kept as control.

### In vitro antibacterial activity of Chitosan-Zn complex

Chitosan (60mg) was dissolved in 6ml acetic acid (1%). Dispensed 1 ml of the above solution into 5 different tubes. Added 4ml, 2ml, 1ml, 0.5ml and 0.25ml of $ZnSO_4.7H_2O$ to the tubes and stirred. The pH is adjusted to 7 by adding 0.1M NaOH. The mixture is refluxed at $80^0$C for 3 hr with stirring, it is then cooled to room temperature. Added 8ml of acetone to each tube. The resulting white precipitate obtained by filtration was repeatedly washed with ethanol and dried under vacuum to constant weight. The complexes, and chitosan solution prepared in 1% acetic acid were autoclaved at $121^0$C for 15 min. Antibacterial activity was done with $ZnSO_4.7H_2O$ as control. The zones of inhibition produced by chitosan-Zn complexes were measured.

## In vitro Antibacterial Activity

All the isolates and ATCC strain were lawn -cultured on MHA plates. Chitosan of different concentrations (100µg, 200µg, 300µg and 400µg) and hydrolysates were added into each well (Ditch-Plate method: *Rice et al*, 1950.) and incubated at $37^0$C for 24hrs. The zone of inhibition was measured in millimeters. Antibacterial activity of chitosan against the given clinical isolates was then compared with the reference standard antibiotics Vancomycin (VA) and Amoxyclav (AC). The minimum inhibitory concentration (MIC) of chitosan and standard antibiotics were measured.

## Results and Discussion

### Antibacterial activity of chitosan

As shown in Table (1, 2 and 3) chitosan markedly inhibited the growth of all the three organisms tested, namely *Staph. aureus,* Coagulase negative *Staph. aureus* and *Enterococci.* The activity increased with increasing concentration of chitosan. The susceptibility of the test organisms to different concentrations of chitosan was strain dependant. The inhibition zone diameter for *Staph. aureus* was in the range of 14-30mm, the highest antibacterial activity (30mm) was against the ATCC strain of *Staph. aureus.* The inhibition zone diameter ranged between 12-22mm for Coagulase negative *Staph. aureus* and 17-29mm for *Enterococci spp.*

The concentration of chitosan used in this study (1%) was same as that used by *Bae et al* (2006) in a clinical trial to study the effect of chitosan on plaque formation. The 1.0%, water-soluble reduced chitosan, with pH ranging from 6.0 to 6.5, molecular weights between 3,000 and 5,000 Da, and 70% degree of deacetylation, showed an MIC of 1.25g/L for *Streptococcus mutans*. The water-soluble reduced chitosan exhibited potent antibacterial effect on *S. mutans,* and displayed a significant antibacterial and plaque-reducing action during the 4-day plaque regrowth. The antibacterial activity of chitosan was tested against two strains of *Staphylococcus aureus* and *Salmonella paratyphi* by *Islam et al* (2011). They found that the highest zone of inhibition against *Salmonella paratyphi* and *Staphylococcus aureus* were 16 mm and 14 mm respectively at the dose of 288µg. The chitosan preparation in our study showed a diameter range of 12-22mm for 200µg, hence more effective. Acetic acid (1%) was taken as control and it showed no antibacterial activity against any of the test organisms.

### Minimum Inhibitory Concentration (MIC)

MIC is usually taken as a measure of the susceptibility of a bacterial strain towards a specific antimicrobial substance. It is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (*Monarul et al, 2011*.) The MIC value of chitosan against the clinical isolates was 100µg.

It is interesting to note that chitosan (400µg) showed the highest activity against *Staph. aureus,* relative to other organisms, as judged by the highest inhibition zone diameter 30mm (Table.1). On the other hand, Coagulase negative *Staph. aureus* exhibited the lowest (12mm) antibacterial activity (Table.2).

### Comparison of the Antibacterial Activity of Chitosan with Standard Antibiotics

Chitosan showed significant antibacterial activity against the clinical pathogens tested.

The antibacterial activity of chitosan (400µg) against ATCC strain of *Staph. aureus* was twice that for Vancomycin and Amoxyclav (Table.1). The antibiotic resistant strains of *Staph. aureus* were also susceptible to chitosan activity. Out of the 17 clinical isolates of *Staph. aureus* tested, 13 strains were comparatively more sensitive to Vancomycin than Amoxyclav. Strains of Coagulase negative *Staph.aureus* did not show chitosan sensitivity like the other two Gram positive pathogens tested (Table.2).

Among the 15 strains of *Enterococci spp* tested, 13 showed remarkable sensitivity to chitosan followed by Vancomycin than Amoxyclav (Table,3).

The use of chitosan as an anti-biofilm coating for medical applications such as implantable medical devices, wound dressings, catheters and contact lenses has been suggested by *Carlson et al (2008)*, who claim that coating surfaces with chitosan is highly effective at retarding or preventing the formation of *Staph. aureus* and *Staph.epidermidis* biofilms. Hypothesis is that it disrupts cell membrane as microbes settle on the surface, being even superior to coatings impregnated with antimicrobial agents such as Chlorhexidine (*Ion Anghel et al*, 2012).

Chitosan shows great potential in developing into a biocompatible antibiotic. Chitosan with a high molecular weight (500KD-1000KD) and maximum degree of de-acetylation is expected to show enhanced antimicrobial activity even at lower concentrations. *Rhodes* and *Roller* (2000); *Raafat* (2008), theorized that a mild degradation of chitosan enhances its antimicrobial action, where as highly degraded chitosan displayed no antimicrobial action.

The observation that the action of chitosan on *Staph. aureus* increased with increase in its concentration is quite evident. The mean values of zone diameters for chitosan concentrations, 100µg, 200µg, 300µg and 400µg were 10.35mm, 14.59mm, 17.7mm and 20.24mm respectively. The level of significance was 0.000, which was within the range of significance (0.00 to 0.05), thus indicating that the increase in concentrations does influence the antibacterial effect of chitosan. The level of significance was the same (0.000) in the case of Coagulase negative *Staph*.

**Antibacterial Activity of Chitosan Derivatives**

Antibacterial Activity of $H_2O_2$ degraded Chitosan Hydrogen peroxide degradation of chitosan has not significantly affected its antibacterial activity against clinical isolates of *Staph.aureus*, except for strain SA-2. The antibacterial activity against SA-2 has reduced by more than 60% after degrading chitosan with hydrogen peroxide. The antibacterial activity of $H_2O_2$ degraded chitosan ranged between 8 -25 mm against *Staph.aureus* (Fig.1).

CoNS also showed similar type of action with $H_2O_2$ degraded chitosan (Fig.2).

But in the case of Enterococci, the most significant antibacterial activity is given by EC-3. The zone diameter for the antibacterial activity is ranged between 11-26mm (Fig.3).

It was previously reported that oxidative destruction of chitosan occurred in presence of hydroxy radicals which were generated from $H_2O_2$ (*Nova et al*.,2001; *Qin et al*.,2002). Therefore, the reduced amount of hydrogen after this reaction may account for the $H_2O_2$- scavenging effect of the chitosan

derivatives observed in their studies. The hydroxyl radicals generated from $H_2O_2$ may react with parts of chitosan molecule other than the β- 1,4 linkage to form oligomers with non-active oxidized groups. It was not possible to establish whether the improvement in the antimicrobial potency of chitosan degraded by $H_2O_2$ was due to variation in the degree of polymerization or to the presence of oxidized groups on the oligomers. It is possible that oxidation increased the biological activity of the polymer, while excessive hydrolysis of the polymeric backbone resulted in a loss of activity ( *Rhodes* and *Roller,2000*).

*Jeon et al* (2001), indicated that native chitosan (685 KDa) was more effective on Gram-positive and Gram-negative bacteria than chitosan oligomers, whose molecular weights are between 1-10 KDa. *No et al* (2002), also reported that native chitosan showed higher antibacterial activity than chitosan hydrolysates. The results we obtained are in agreement with the above findings as, in the case of *S.aureus*. The zone diameter decreased with increase in concentration in the mixture. This is because, in the case of Gram- positive *cocci*, degraded chitosan shows lesser antibacterial activity than that of native chitosan.

*Enterococcal* strains showed the greatest zone size than for *S.aureus* and Coagulase negative *staph* tested. In contrast to the earlier studies, Uchida *et al* (1989), found that chitosan hydrolysates, slightly hydrolyzed by chitosanase, was more effective as an antibacterial agent than native chitosan. The limited exposure of chitosan (2hrs) to iron might have partially hydrolyzed chitosan thereby expressing enhanced activity. The activity might also be due to the formation of free radicals on the hydrolysates.

**Antibacterial Activity of Lysozyme degraded Chitosan**

Of the five strains from each group of organisms namely expand; the highest activity is shown by *Enterococci*, then Coagulse negative *Staph* and *Staph.aureus*. The inhibition zone diameter varies between 10 -20 mm (Fig.7).

Nordtveit *et al,(1996)* reported that, lysozyme from chicken egg used for chitosan degradation has been shown to be the most efficacious when the chitosan is only partially deacetylated. Antimicrobial activity of hydrolyzed chitosan (473-583KDa) against pathogenic *E.coli* was not different from the native chitosan (438 KDa), where as the inhibition against *S.aureus* and *C.albicans* was lower than the native one. The chitosan molecular weight and antimicrobial activity from the enzymatic study were different from the previous result due to the limitation of chitosan solubility in the buffer (instead of 1% acetic acid), which was used to facilitate the lysozyme activity. Lysozyme could be effective against Gram-negative bacteria when outer membranes of bacteria are disrupted or uncovered by chitosan.

**Antibacterial Activity of Chitosan- Zinc complex**

A single strain of each organism was studied in triplicate to assess the antibacterial effect of CS-Zn complexes, all the chelates had more action than CS and Zn for *S. aureus* (ATCC 25923) but the CoNS and EC *spp* showed a decrease in activity than chitosan for ratios 0.25:1, 0.5:1 and 1:1. As concentration of Zn increases, the zone diameter also increases. The antibacterial activity of CS-Zn complexes enhanced with increasing chelate ratios, for all the three organisms.

**Table: 1.** Antibacterial activity of chitosan and standard antibiotics on *Staphylococcus aureus* strains

| ***Staphylococcus aureus* strains** | **Clearance zone diameter in mm of Chitosan** | | | | **Vancomycin**(Va) | **Amoxyclav**(Ac) |
|---|---|---|---|---|---|---|
| | 100µg | 200µg | 300µg | 400µg | | |
| ATCC(25923) | 16 | 22 | 28 | 30 | 15 | 15 |
| SA-1 | 18 | 22 | 26 | 28 | 16 | 33 |
| SA-2 | 0 | 6 | 8 | 15 | 16 | 17 |
| SA-3 | 15 | 20 | 24 | 27 | 19 | 18 |
| SA-4 | 10 | 16 | 18 | 20 | 18 | 21 |
| SA-5 | 10 | 14 | 17 | 19 | 15 | 11 |
| SA-6 | 10 | 14 | 17 | 20 | 12 | 8 |
| SA-7 | 10 | 14 | 18 | 19 | 0 | 0 |
| SA-8 | 16 | 19 | 23 | 25 | 18 | 20 |
| SA-9 | 16 | 20 | 23 | 27 | 15 | 12 |
| SA-10 | 14 | 17 | 20 | 22 | 17 | 17 |
| SA-11 | 6 | 9 | 13 | 16 | 17 | 17 |
| SA-12 | 7 | 10 | 12 | 14 | 15 | 30 |
| SA-13 | 6 | 10 | 12 | 14 | 15 | 11 |
| SA-14 | 7 | 12 | 14 | 16 | 16 | 13 |
| SA-15 | 6 | 11 | 14 | 16 | 16 | 14 |
| SA-16 | 9 | 14 | 15 | 16 | 19 | 0 |

SA= *Staphylococcus aureus*

fig:1.Antibacterial activity of $H_2O_2$ degraded chitosan against SA

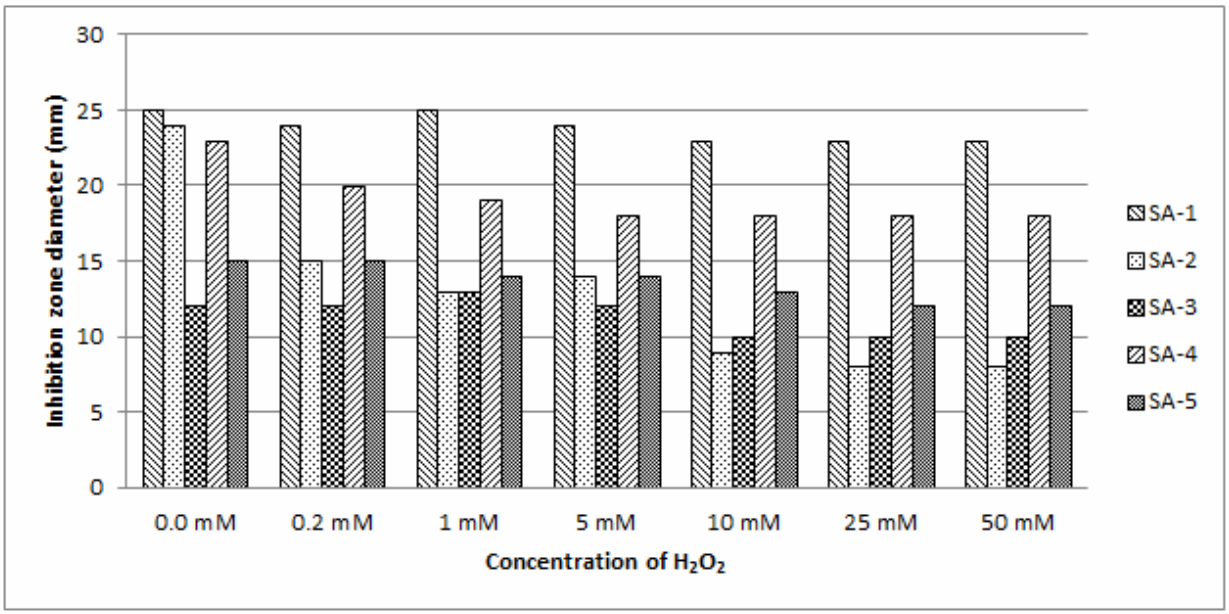


**Table.2** Antibacterial activity of chitosan and standard antibiotics on Coagulase negative *Staphylococcus aureus* strains

| **Coagulase negative *Staphylococcus aureus* strains** | **Clearance zone diameter in mm of chitosan** | | | | **Vancomycin**(Va) | **Amoxyclav**(Ac) |
|---|---|---|---|---|---|---|
| | 100µg | 200µg | 300µg | 400µg | | |
| CoNS-1 | 7 | 14 | 17 | 22 | 19 | 0 |
| CoNS-2 | 9 | 12 | 15 | 16 | 15 | 16 |
| CoNS-3 | 7 | 10 | 15 | 19 | 21 | 19 |
| CoNS-4 | 10 | 12 | 14 | 16 | 10 | 10 |
| CoNS-5 | 10 | 14 | 16 | 18 | 20 | 18 |
| CoNS-6 | 10 | 15 | 18 | 20 | 22 | 20 |
| CoNS-7 | 7 | 10 | 12 | 14 | 18 | 20 |
| CoNS-8 | 0 | 7 | 10 | 12 | 17 | 0 |
| CoNS-9 | 6 | 8 | 12 | 14 | 18 | 0 |
| C0NS-10 | 10 | 12 | 15 | 16 | 16 | 0 |
| CoNS-11 | 11 | 12 | 14 | 15 | 12 | 0 |
| CoNS-12 | 7 | 9 | 12 | 13 | 17 | 0 |
| CoNS-13 | 10 | 12 | 16 | 17 | 16 | 18 |
| CoNS-14 | 9 | 11 | 15 | 17 | 17 | 19 |
| CoNS-15 | 8 | 10 | 13 | 16 | 18 | 0 |

CoNS= Coagulase negative *Stahylococcus aureus*

**Table.4** Antibacterial activity of chitosan and standard antibiotics on *Enterococci* strains

| ***Enterococcus* strains** | **Clearance zone diameter in mm of chitosan** | | | | **Vancomycin**(Va) | **Amoxyclav**(Ac) |
|---|---|---|---|---|---|---|
| | 100µg | 200µg | 300µg | 400µg | | |
| EC-1 | 6 | 10 | 16 | 19 | 16 | 20 |
| EC-2 | 19 | 22 | 24 | 26 | 17 | 20 |
| EC-3 | 10 | 14 | 18 | 19 | 0 | 8 |
| EC-4 | 9 | 16 | 20 | 25 | 18 | 25 |
| EC-5 | 11 | 14 | 19 | 23 | 16 | 20 |
| EC-6 | 19 | 21 | 25 | 29 | 20 | 28 |
| EC-7 | 18 | 25 | 27 | 29 | 17 | 17 |
| EC-8 | 12 | 16 | 18 | 20 | 17 | 20 |
| EC-9 | 9 | 15 | 18 | 20 | 15 | 17 |
| EC-10 | 12 | 16 | 21 | 26 | 24 | 28 |
| EC-11 | 10 | 14 | 17 | 18 | 15 | 17 |
| EC-12 | 7 | 10 | 15 | 18 | 15 | 19 |
| EC-13 | 12 | 15 | 18 | 19 | 18 | 21 |
| EC-14 | 10 | 14 | 17 | 20 | 19 | 22 |
| EC-15 | 8 | 12 | 14 | 17 | 18 | 20 |

EC= *Enterococci*

fig:2. Antibacterial activity of $H_2O_2$ degraded chitosan against CoNS

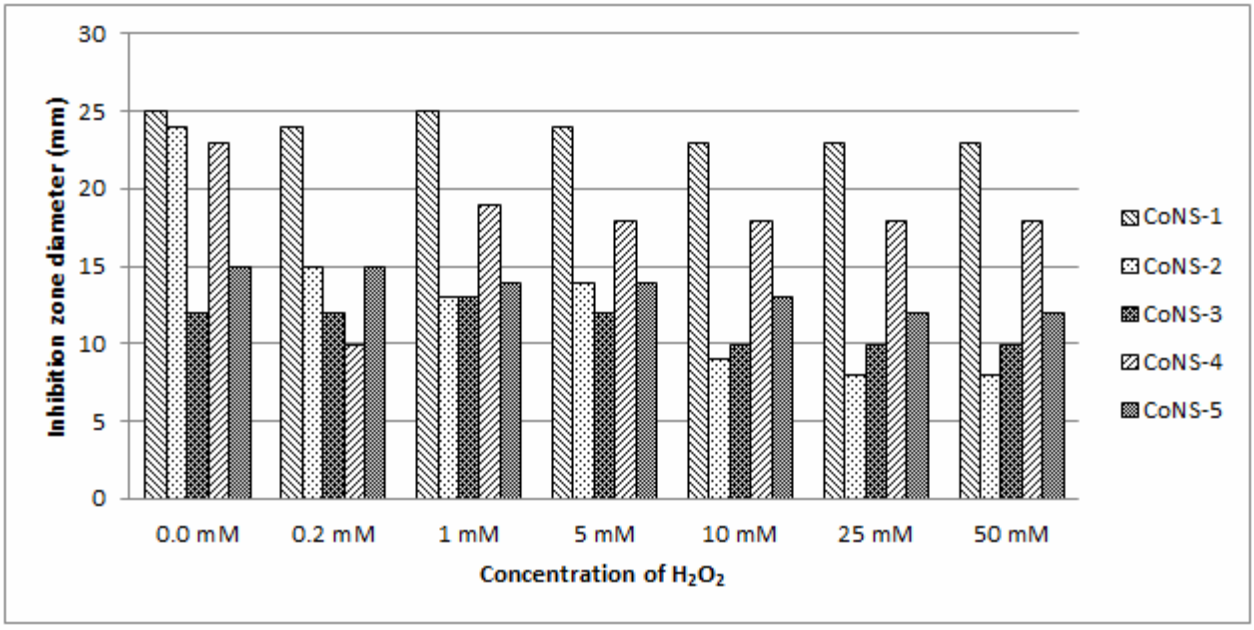



fig:3.Antibacterial activity of $H_2O_2$ degraded chitosan against EC

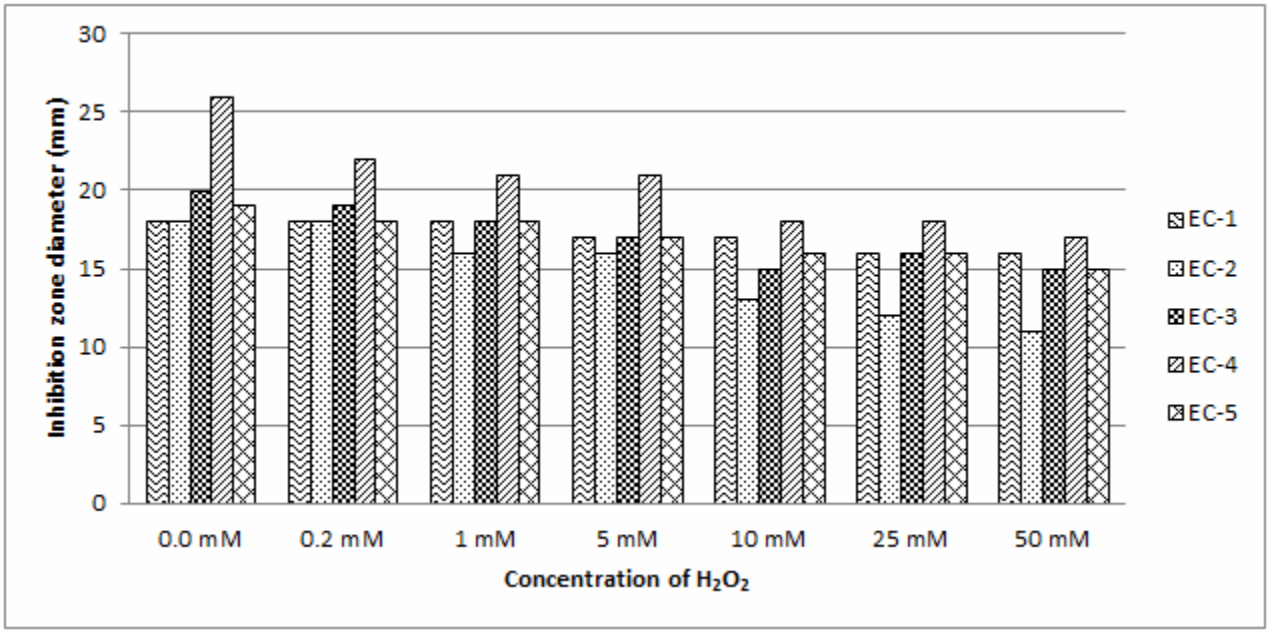


fig:4. Antibacterial activity of lysozyme degraded chitosan against SA, CoNS and EC

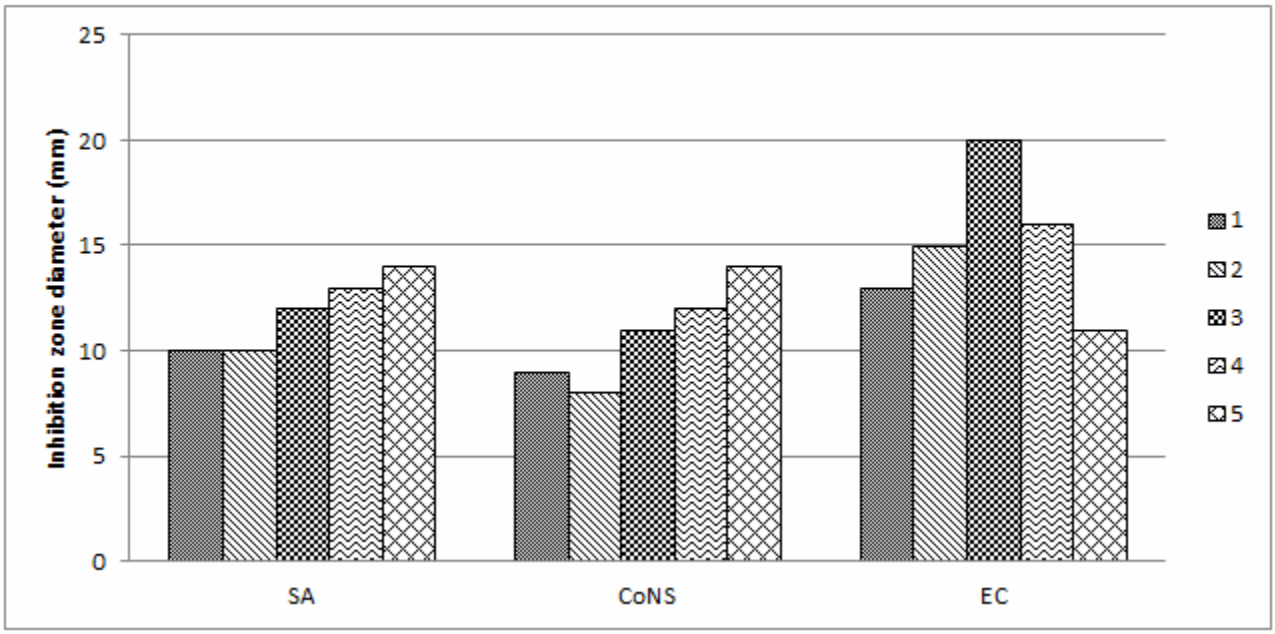
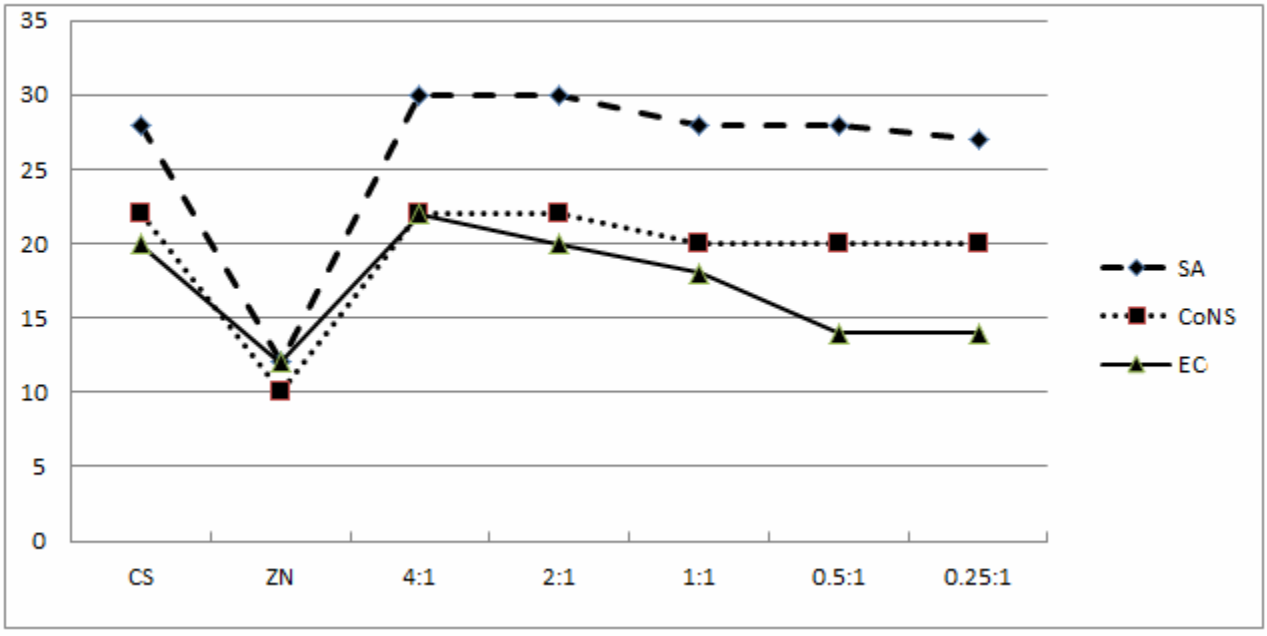



fig:5. Antibacterial activity of CS-Zn complex against SA, CoNS and EC




The control (1% ZnSO4) developed zone of inhibition. The complex with chelate ratio,4:1 was more effective than CS and Zn alone by 2-10 units and 18-20 units respectively (Fig.8). The CS-Zn complex showed a wide spectrum of effective antimicrobial activities (Wang et al,2004), which were 2-8 times and 4-16 folds higher than those of chitosan and zinc sulfate, respectively, and improved with increasing content of zinc ions. The CS-Zn complexes exerted a more effective antibacterial

activity equally against *E.coli* and *Corynebacteria* than antifungal activity (Wang *et al*, 2004).

Although the exact antimicrobial mechanism of chitosan is still unclear, several mechanisms have been proposed. One of the major proposals is that the polycationic nature of chitosan reacts with the negatively charged residues of macromolecules at the cell surface (Young and Kauss, 1983), which could alter the permeability characteristics of chitosan, resulting in the chitosan induced leakage of bacterial cell components, specifically the β(1,4) linkage between C-1 of N- acetyl muramic acid and C-4 of N- acetyl glucosamine (Masschalck and Michiels, 2003). The non-inhibitory activity of glucosamine supports the fact that a large number of protonated amino groups are required for the antimicrobial activity of chitosan, which might not be present in sufficient quantity in equal as even high concentrations of glucosamine than native chitosan.

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