Chitosan Nanoparticles as an Effective Antimicrobial Agents against Gastrointestinal Tract Pathogens

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Abstract - Gastrointestinal tract infections caused by pathogenic microorganisms are a major public health concern worldwide. The development of alternative antimicrobial agents that can effectively control these pathogens while minimizing the risk of drug resistance is urgently needed. In recent years, chitosan nanoparticles (CSNPs) have emerged as a promising candidate for this purpose. This study aimed to evaluate the effectiveness of CSNPs as an antimicrobial agent against selected gastrointestinal tract pathogens, including *Escherichia coli* and *Salmonella typhimurium*. CSNPs were prepared by ionic gelation, and their physicochemical properties, including size, zeta potential, and morphology, were characterized using zetasizer and transmission electron microscopy.

The results showed that CSNP had a mean size of 220nm, a positive zeta potential of 47.0 mV, and a spherical morphology. The CSNPs exhibited excellent antibacterial activity against the tested pathogens, with minimum inhibitory concentrations (MICs) ranging from 0.25 to 1 mg/mL. Moreover, the CSNPs demonstrated a dose-dependent bactericidal effect against all pathogens, with bacterial growth inhibition of up to 99.9% at a concentration of 5 mg/mL.

The results of this study suggest that CNPs are effective antimicrobial agents against gastrointestinal tract pathogens, and their use can potentially minimize the risk of drug resistance. CSNPs can be incorporated into various food and pharmaceutical products to improve their antimicrobial properties and extend their shelf life. Further studies are needed to explore the potential of CNPs as an alternative to conventional antimicrobial agents and to investigate their safety and efficacy in vivo. CSNPs hold significant potential for the development of novel therapeutic strategies to combat GIT infections and mitigate the global burden of gastrointestinal diseases.

I. INTRODUCTION

The emergence of antimicrobial resistance poses a significant challenge to public health worldwide. In recent years, there has been a growing interest in exploring alternative antimicrobial agents to combat the rise of drug-resistant pathogens [1]. Among these alternatives, chitosan nanoparticles have gained considerable attention due to their unique properties and potential applications in various biomedical fields, including the management of gastrointestinal tract infections.

Chitosan, a biodegradable and biocompatible natural hydrophilic polymer derived from chitin, is obtained primarily from crustacean shells. It possesses several desirable characteristics, such as non-toxicity, biocompatibility, and

excellent antimicrobial properties. Moreover, chitosan can be easily processed into nanoparticles with high surface area-to-volume ratio, enhanced stability, and controlled release capabilities, making it an ideal candidate for drug delivery systems and antimicrobial applications [2].
The antimicrobial mechanisms underlying the effectiveness of chitosan nanoparticles (CSNPs) against gastrointestinal (GI) tract pathogens are multifaceted. Firstly, the positively charged amino groups of chitosan interact with the negatively charged microbial cell membranes, leading to membrane disruption and subsequent leakage of cellular components [3]. Secondly, CSNPs can induce oxidative stress within microbial cells by generating reactive oxygen species (ROS), impairing their vital cellular functions and ultimately resulting in microbial death [4]. Furthermore, CSNPs possess mucoadhesive properties, allowing them to interact with the mucus layer lining of the GI tract, which aids in preventing bacterial adhesion and subsequent colonization [5].

The gastrointestinal tract is a complex ecosystem that houses various microorganisms, including commensal bacteria and potential pathogens. Disruption of the normal microbial balance can lead to the colonization and proliferation of pathogenic bacteria, causing infections and gastrointestinal disorders. Common pathogens associated with gastrointestinal infections include *Escherichia coli*, *Salmonella spp*., *Campylobacter spp*., and *Helicobacter pylori* [6].

Recent studies have also investigated the antimicrobial efficacy of chitosan nanoparticles against a range of gastrointestinal pathogens. An *in vitro* study to evaluate the antimicrobial activity of chitosan nanoparticles against *Salmonella enterica*. The results demonstrated that chitosan nanoparticles effectively inhibited the growth of Salmonella enterica by disrupting the bacterial cell membrane integrity, resulting in bacterial cell death [7].

Furthermore, other research by explored the potential of chitosan nanoparticles as antimicrobial agents against *Helicobacter pylori*. The study demonstrated that chitosan nanoparticles exhibited potent antibacterial activity against *Helicobacter pylori* strains, effectively suppressing their growth and biofilm formation. The mechanism of action was attributed to the strong affinity of chitosan nanoparticles for bacterial cell surfaces, leading to membrane disruption and subsequent cell death[8]. Based on previous research finding the current research article aims to provide the findings of the antimicrobial efficacy of chitosan nanoparticles against *Escherichia coli* and *Salmonella typhi*. These findings highlight the promising antimicrobial potential of chitosan nanoparticles in combating gastrointestinal tract pathogens. By targeting the specific microbial pathogens while minimizing the impact on commensal bacteria, chitosan nanoparticles offer a potentially safer and more effective approach for the treatment and prevention of gastrointestinal infections.

II. MATERIALS AND METHODS

A. *Materials:*

Low molecular weight chitosan (LMW, 50–190 kDa, Degree of deacetylation; DD = 75–85%), tripolyphosphate (TPP), and acetic acid were purchased from Sigma-Aldrich. Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) were obtained from Oxoid™). All the reagents were prepared in double deionized autoclaved and filtered water. Glassware was washed with detergents, autoclaved and oven dried, prior to the use in experimentation. All the other solvents and chemicals were of analytical grade. The antimicrobial susceptible disc catridges (Imipenem, Erythromycin, Ampicillin, Gentamycin and Nitrofurantoin) were obtained from Oxoid™.

B. *Methods:*

1. *Synthesis of the CSNPs*

The chitosan nanoparticles (CNPs) were synthesized using the ionic gelation method, following a previously described protocol with slight modifications [9, 10]. Low molecular weight (LMW) chitosan solutions were prepared at concentrations of 1, 2.5, and 5 mg/mL by dissolving 0.01, 0.025, and 0.05 g of chitosan, respectively, in 10 mL of a 1% v/v acetic acid solution. Subsequently, 1 mL of a 0.1% w/v TPP solution was added to 4 mL of the chitosan solution while continuously stirring at 1000 rpm for 1 hour, resulting in spontaneous formation of the nanoparticles. The nanoparticle solution was then subjected to centrifugation at 15,000 × g for 20 minutes, and the

obtained nanoparticles were washed twice by suspending them in distilled water followed by additional centrifugation steps. Finally, the CNPs were reconstituted in deionized distilled water for further characterization and experiments, and they were stored at 4°C.

2. Characterization of CSNPs

The chitosan nanoparticles (CSNPs) were characterized using Fourier Transform Infrared Spectroscopy (FTIR) Nicolet iS5,Thermo Electron Scientific, US, to analyze their structural composition and confirm the presence of functional groups. A small amount of dried CSNPs was collected and finely ground to obtain a homogeneous powder. The powder was then placed in a sample holder, ensuring a thin and uniform layer and was analyzed using a Fourier Transform Infrared Spectrometer. The CSNPs sample holder was inserted into the spectrometer, and the instrument was calibrated for baseline correction and background noise reduction. The FTIR spectra of the CSNPs were obtained by scanning the sample in the mid-infrared region (4000-400 cm^{-1}). The characteristic peaks in the FTIR spectra were assigned to various functional groups of chitosan, such as hydroxyl (OH) stretching, amine (NH) stretching, carbonyl (C=O) stretching, and glycosidic linkages.

3.Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of *Escherichia coli* (E. coli) and *Salmonella typhi* (S. typhi) isolates was conducted using the agar dilution method, following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, et al., 2020). The tested antibiotics included ampicillin, imipenem, erythromycin, gentamicin, and nitrofurantoin. Various concentrations of these antibiotics were incorporated into the Mueller-Hinton Agar (MHA) medium. The results of the antibiotic susceptibility testing, including the MIC values and interpretation of susceptibility, were reported in the research article.

4. Minimum Inhibitory Concentration of CSNP

The minimal inhibitory concentration (MIC_{90}) and minimal bactericidal concentration (MBC) of chitosan nanoparticles (CNPs) were determined using broth microdilution assays. CSNPs were diluted in MHB broth medium with a pH adjusted to less than 6. Serial twofold dilutions of CSNPs ranging from 2.5 mg/mL to 0.016 mg/mL were prepared in 100 well microtiter plates. Each well contained a total volume of 100 µL. Bacterial cell suspensions were adjusted to a McFarland 0.5 standard, corresponding to a bacterial density of 1-2 × 10^8 colony-forming units (cfu) per mL. The suspension was further diluted to achieve a final inoculum density of 5 × 10^5 cfu/mL in the microdilution panels.The microtiter plates were incubated for 24 hours at 37°. This allowed for bacterial growth in the presence of CSNPs. The optical density (OD) of bacterial growth in each well was recorded at 600nm.

5. Scanning Electron Microscopy

Bacterial inoculum was prepared and subjected to treatment with CSNP. Following treatment, the samples were incubated for a period of 20-24 hours to allow for bacterial growth and interaction with the nanoparticle. The bacterial cells were carefully washed using phosphate buffer saline (PBS) to remove any residual compounds. Control cells, which were not exposed to any treatment, were also included for comparison purposes. To prepare the bacterial culture for analysis, it was fixed with a solution containing 2% glutaraldehyde in PBS for a duration of one hour. Subsequently, the samples underwent additional fixation using 1% osmium tetraoxide for one hour. Following fixation, the samples were once again washed with PBS to ensure proper cleaning and removal of fixatives. Next, the supernatant was carefully removed, and the samples were subjected to a dehydration process using a series of graded ethanol solutions. This step helps to remove water from the samples while preserving their structural integrity. The dehydrated specimens were mounted onto stubs and coated with a conductive metal layer using an ion sputtering device. This coating enhances the samples' conductivity and allows for optimal imaging in the scanning electron microscope. Finally, the coated samples were examined and analyzed using a scanning electron microscope, enabling the visualization of the bacterial cells and the assessment of their morphology and surface characteristics [11].

III- EXPERIMENT AND RESULT

1. Functional Bond Analysis by FTIR:

The infrared spectroscopy analysis revealed specific characteristic bands of chitosan as shown in Figure 1. The presence of the characteristic band at 3466 cm-1 indicated the stretching of the OH bond, while the band at 2941 cm-1 corresponded to the stretching vibration of NH_2 functional groups in chitosan. The asymmetric stretching vibration of CH_2 was observed at 2920 cm-1. Further analysis identified the presence of the amide-I band at 1641 cm-1, indicating the successful derivatization of chitin into chitosan during the deacetylation process. The vibration of amide-III was observed at 1112 cm-1. The OH and CH vibrations were found at 1465 cm-1. The characteristic bands corresponding to the stretching of C-O-C bridge, indicative of polysaccharide (glycosidic) linkages, were observed at 617 cm-1. The presence of the wave number at 617 cm-1 in the fingerprinting region suggested the ring stretching of β (1→4) glycosidic linkage, a characteristic linkage of polysaccharides present in chitosan.

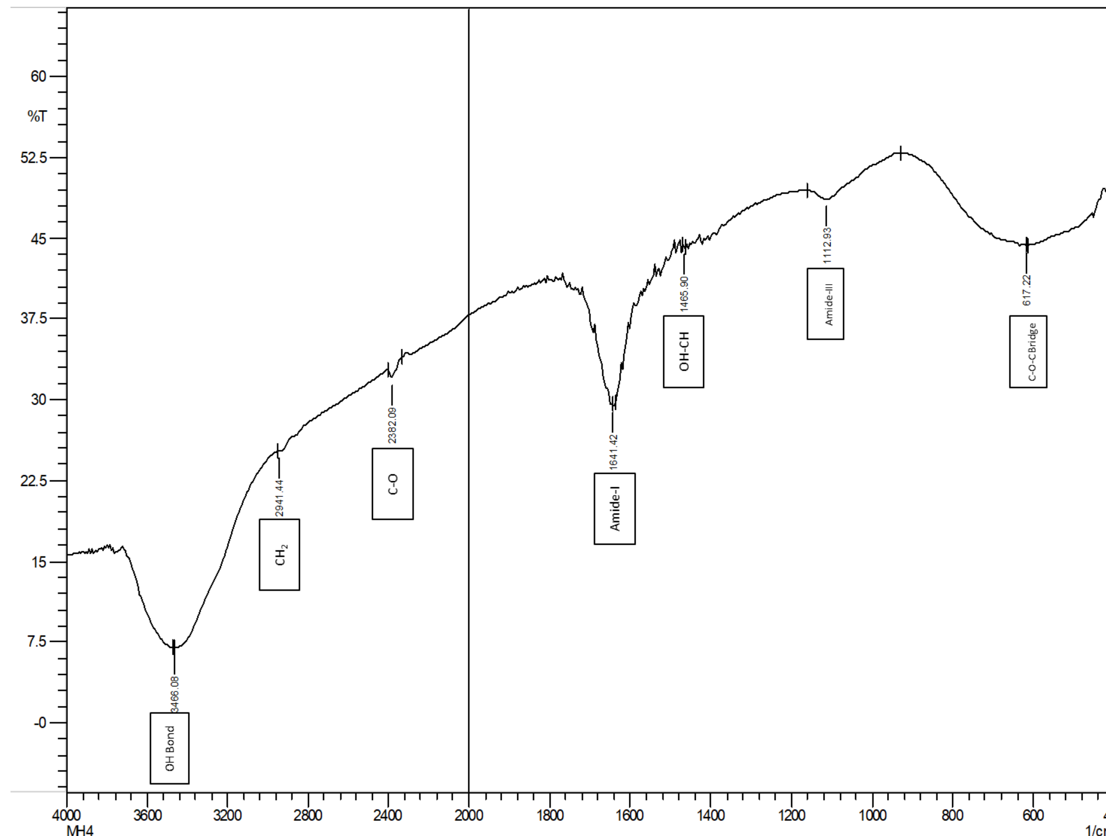

Figure 1: Functional Bond Analysis of CSNP through FTIR with characteristic peak showing the presence of chitosan nanoparticles.

2. Particle Size Distribution by Zetasizer

The chitosan nanoparticles were fabricated at concentration 5mg/ml resulting in a particle size of 220nm. The zeta potential of the nanoparticles was measured to be +47mV, indicating a positive surface charge. The polydispersity index (PDI) value of the nanoparticles was determined to be 0.44, suggesting a relatively narrow size distribution (Figure 2). The decision to select these chitosan nanoparticles for antibacterial susceptibility testing was influenced by their small particle size, which was achieved during the fabrication process. The smaller particle size is often desirable as it can enhance the nanoparticles' cellular uptake and potential biological activity. These specific chitosan nanoparticles with their favorable particle size, positive zeta potential, and relatively narrow size distribution were deemed suitable for further evaluation of their antimicrobial analysis against GIT pathogens. The results showed that at 5mg/ml, the zeta potential was measured to be +47 mV which showed that chitosan nanoparticles exhibited a cationic nature, as their zeta potential values were above +30 mV. This aligns with previous literature, which suggests that nanoparticles synthesized using low molecular weight chitosan tend to exhibit zeta potential values above +30 mV. The cationic nature of the chitosan nanoparticles contributes to their high stability as colloidal

solutions and makes them less prone to aggregation or conglomeration [12]. Therefore, the observed zeta potential values of the fabricated chitosan nanoparticles confirm their cationic nature and suggest that they possess good colloidal stability, which is advantageous for their potential applications in medicinal fields.

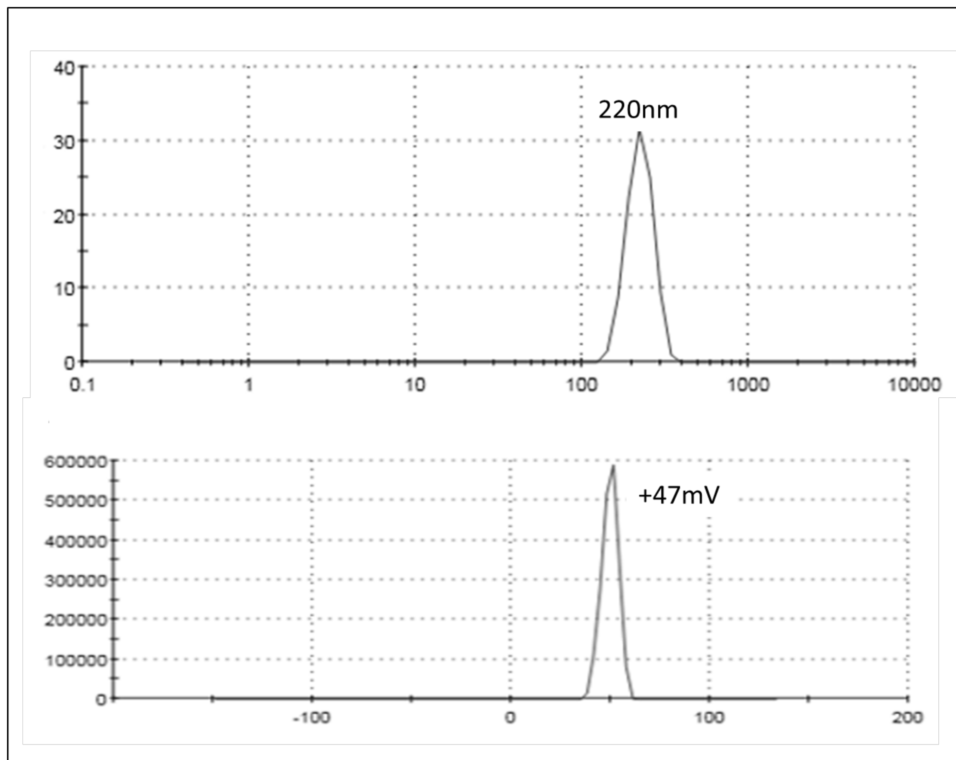

Figure 2: Polydispersity Index and Zeta potential of CSNP at 5mg/ml showing the particle size and Zeta potential

3. Antimicrobial Susceptibility Testing against CSNP

The results presented in the Table-1 demonstrate that the antibiotic imipenem exhibited the highest zone of inhibition, with measurements of 30mm and 20mm against the respective pathogens, resulting in an inhibition of approximately 80% of bacterial growth. Similarly, gentamicin and nitrofurantoin displayed moderate sensitivity towards the pathogens. In contrast, erythromycin and ampicillin demonstrated significant resistance against both strains. In order to check the alternative of these effective antibiotics, we tested their efficacy with chitosan nanoparticles at various concentrations, which yielded improved outcomes. Our findings highlight the potential of chitosan nanoparticles as a promising strategy to augment the activity of antibiotics and improve their susceptibility against gastrointestinal tract pathogens.

The Table-2 presents the results of the antimicrobial activity of chitosan nanoparticles (CSNPs) at different concentrations against *E.coli* and *S.typhi*. At a concentration of 1 mg/mL, the CSNPs exhibited a zone of inhibition of 10mm against *E.coli* and 30mm against *S.typhi*. When the concentration was increased to 2.5 mg/mL, the zone of inhibition expanded to 25mm for *E.coli* and 45mm for *S.typhi*, indicating a significant increase in antimicrobial activity. At a concentration of 5 mg/mL, the CSNPs demonstrated a slightly larger zone of inhibition of 30mm for *E.coli* and 40mm for *S.typhi*. From the table, it can be observed that as the concentration of CSNPs increased, there was a corresponding increase in the zone of inhibition, suggesting a concentration-dependent antimicrobial effect. These findings highlight the potential of CSNPs as effective agents against *E.coli* and S. typhi, with higher concentrations leading to greater antimicrobial activity.

These findings emphasize the significance of chitosan nanoparticle concentration in determining their antimicrobial activity against gastrointestinal tract pathogens, offering valuable insights for the development of effective antimicrobial agents with decreased resistance

Antibiotics	Zone of inhibition	
	E.coli	*S.typhi*
Imipenem	30mm	20mm
Erythromycin	0.20mm	0.30mm
Ampicillin	0.25mm	0.10mm
Gentamycin	15mm	20mm
Nitrofurantoin	20mm	25mm

Table 1: Antibiotic Sensitivity Testing against GI Pathogens

Concentration of CSNP	Zone of inhibition	
	E.coli	*S.typhi*
1 mg/ml	10mm	30mm
2.5 mg/ml	25mm	45mm
5mg/ml	30mm	40mm

Table 2: Varying concentration of Nanoparticles against GI Pathogens

4. Minimum Inhibitory Concentration (MIC)

The Table-3 revelaed the results of the minimum inhibitory concentration (MIC90) and minimum bactericidal concentration (MBC) of chitosan nanoparticles (CSNPs) at different concentrations against *E. coli* and *S. typhi* strains. The CSNPs were tested at concentrations of 5mg/ml, 2.5mg/ml, and 1mg/ml. For E. coli strain, the MIC90 values of CSNPs were 0.30, 0.25, and 0.10 for concentrations of 5mg/ml, 2.5mg/ml, and 1mg/ml, respectively. The MBC values were 0.62, 0.30, and ND (not determined) for the corresponding concentrations.

In the case of S. typhi strain, the MIC90 values of CSNPs were 0.45, 0.40, and 0.30 for concentrations of 5mg/ml, 2.5mg/ml, and 1mg/ml, respectively. The MBC values were 0.72, 0.64, and 0.52 for the same concentrations.

Overall, the results indicate that the efficacy of the CSNPs against both E. coli and S. typhi strains varied depending on the nanoparticle concentration. Higher concentrations generally exhibited lower MIC90 values, indicating a stronger inhibitory effect on bacterial growth. The MBC values also demonstrate the concentration-dependent bactericidal activity of the CSNPs.

These findings suggest that chitosan nanoparticles have the potential to be effective antimicrobial agents against gastrointestinal tract pathogens such as E. coli and S. typhi, with their efficacy influenced by the concentration of the nanoparticles

E. coli *Strain*	CSNP (5mg/ml)	CSNP (2.5mg/ml)	CSNP (1 mg/ml)	*S.typhi* *Strain*	CSNP (5mg/ml)	CSNP (2.5mg/ml)	CSNP (1 mg/ml)
MIC_{90}	0.30	0.25	0.10	MIC_{90}	0.45	0.40	0.30
MBC	0.62	0.30	ND	MBC	0.72	0.64	0.52
Imipenem (Standard Drug) MIC_{90} = I							

Table 3: Minimum Inhibitory Concentration of CSNP at different Concentrations

5.Scanning Electron Microscopy:

SEM was employed to evaluate the three-dimensional morphology of both untreated and treated bacterial cells. The SEM images presented in Figure 3A depict the morphology of untreated *Escherichia coli* clinical isolate cells, which remained stable and intact. The cells exhibited smooth surfaces and maintained their original shape and average length. While Figure 3B showed the cells treated with CSNP at concentration 5mg/ml against *E.coli*. It was resulted

in a noticeable effect on the cellular morphology of the strain, with observed ruptured cell membranes and leakage of intracellular content. The treated cells showed alterations in structural integrity and flaccidity, indicating cell death caused by the lethal effects of the nanoparticle.

Similarly, Figure 3C SEM image showed a *Salmonella typhi* clinical isolate strain, displaying normal cellular morphology with intact structural integrity. In contrast, Figure 3D shows complete rupturing of the cell membrane, indicating disintegration of structural stability and leakage of intracellular content when treated with CSNP at 5mg/ml.

These SEM images provide valuable visual evidence of the morphological changes induced by the treatment of bacterial cells with CSNP.

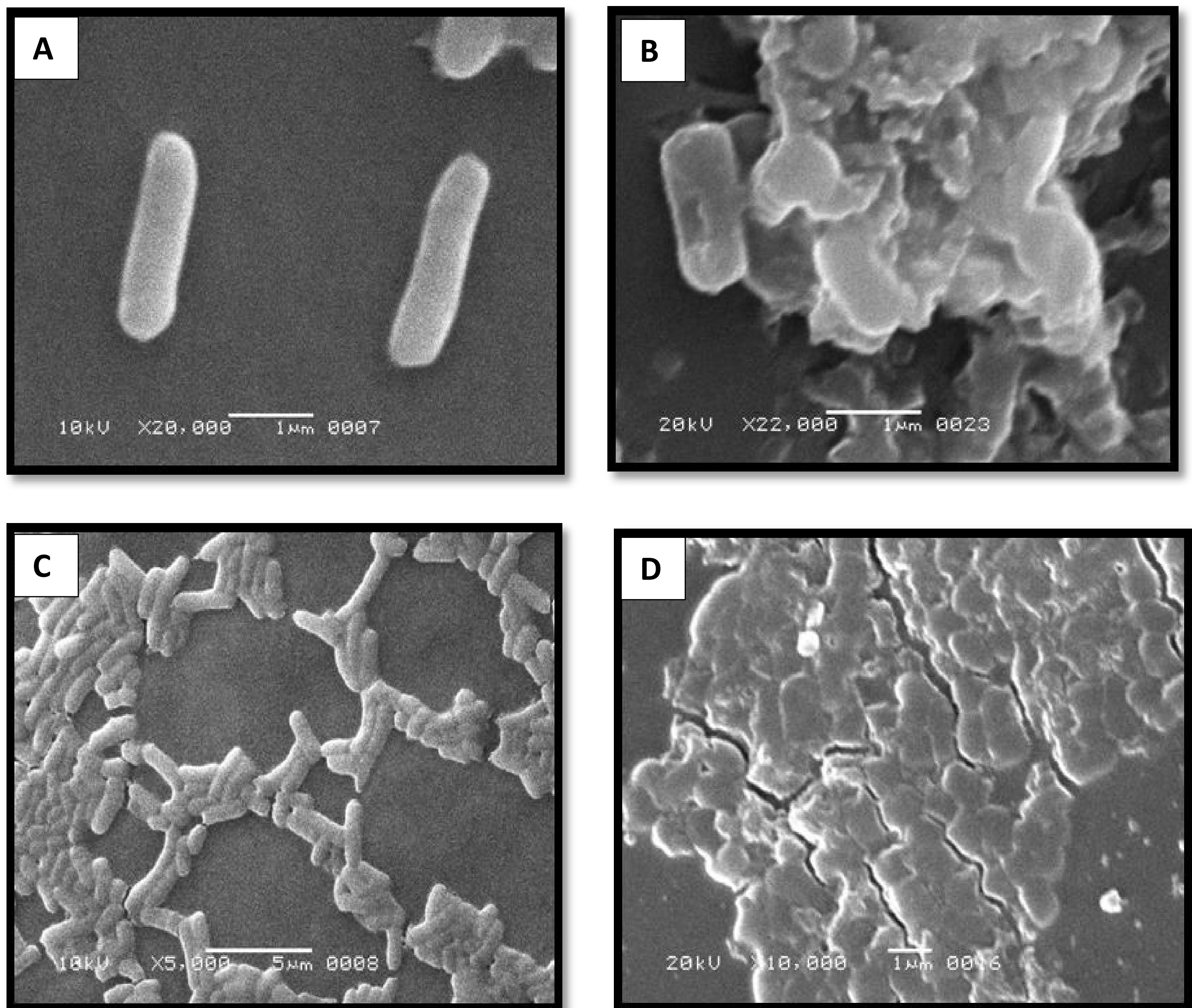

Figure 3: Antibacterial Efficacy of Chitosan Nanoparticles: A). Control untreated cell of *Escherichia coli*, B). CSNP at 5mg/ml against *E.coli*, C). Control untreated cell of *Salmonella typhi*, D). CSNP at 5mg/ml against S.typhi

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