Original Article

The Antimicrobial Activity of Nanochitosan and Nano-$CaCO_3$ against Some Bacteria

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Abstract

Background: The growing threat of infections and drug-resistant microorganisms is a crucial challenge; hence, finding novel antimicrobial medicines is urgently needed. Nanotechnology has garnered interest in many disciplines, especially for therapeutic applications. Chitosan nanoparticles (CS-NPs) and calcium carbonate nanoparticle ($CaCO_3$-NP) are recognized as antimicrobial agents because of their antimicrobial features and minimal risk of toxicity to humans. **Objectives:** The goal of this investigation was to detect the antibacterial efficacy of CS-NPs and $CaCO_3$-NP at various concentrations toward different bacteria. **Materials and Methods:** This investigation collected a total of 128 different clinical specimens. Every bacterial isolate was examined using the cultural, microscopic, and biochemical procedures. Antibiotic sensitivity was performed by using disk diffusion methods. The antibacterial activity of different CS-NP and $CaCO_3$-NP concentrations (20, 40, 60, and 80 mg/L) was estimated on medical bacteria, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*, and by well diffusion method. **Results:** A total of 80 bacterial isolates were collected from various clinical samples. The majority of isolates were *P. aeruginosa*, *E. coli*, *S. aureus*, *S. pyogenes*, and *K. pneumoniae*. Most isolates exhibited resistance against tested antibiotics, in which *P. aeruginosa* exhibited relatively elevated resistance to mainly used antibiotics. Nanoparticle compounds exhibited antimicrobial activity at different concentrations against all bacteria, and it is affected in different degrees. As nanoparticle concentrations increase, antibacterial activity increase as well. **Conclusions:** CS-NPs and $CaCO_3$-NP showed promising antimicrobial activity against medically relevant microorganisms. It is indicated as an option the manufacturing of antimicrobial medications utilized in medicine.

Keywords: Antimicrobial activity, chitosan, nanocalcium carbonate, nanochitosan, nanoparticles

Introduction

Microorganisms are the major cause of infections and infection-derived fatal diseases.[1] The risk of mortality linked to microbial infections keeps rising despite improved understanding of microbial pathogenesis and the use of new therapeutics.[2] Both Gram-positive (G+) and Gram-negative (G−) bacterial species are commonly considered to be critical hazards to public health. For a long period, antibiotics have been utilized to treat illnesses.[3] Antibiotic-resistant bacteria are now a major global problem due to the widespread indiscriminate use of antimicrobial medications used to treat infectious illnesses.[4] Various bacterial pathogens have evolved resistance in recent years, including multidrug-resistant strains of *Staphylococcus aureus*, *P. auroginosa*, and *Escherichia coli*[5]; therefore, researchers are trying to find new antibacterial substances that are nontoxic, eco-friendly, and display strong and wide antimicrobial action with persistent effect.[6]

Chitosan has lately gained interest due to its good antibacterial characteristics and the benefit of being nontoxic, biocompatible, and biodegradable.[7] It is a semisynthetic evolved from chitin, the second prevalent

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polymer that occurs in nature.[8,9] It is a biopolymer made when chitin, a key component of the exoskeleton of insects and crustaceans including crab, shrimp, and crawfish, is alkaline deacetylated. Chains of N-acetyl-D-glucosamine and D-glucosamine monomeric units make up chitosan. These chains are connected by (1–4) glycosidic linkages.[10] Because of the amino groups included in its construction, chitosan has a positive character that, in reduced pH environments, provides chitosan bioactivity by allowing it to interact with substances with negative charges found in bacterial cells, such as polysaccharide, phospholipids, and proteins. Chitosan nanoparticles (CS-NPs) combine the benefits of nanoparticles with the characteristics of chitosan.[11] CS-NPs exhibited significant antimicrobial efficacy on many kinds of microorganisms, involving bacteria and fungi.[12] The large surface-to-volume proportion was the most significant feature of nanoparticles.[13] Nanochitosan demonstrated better antibacterial activity than chitosan solutions, the nanoparticles and smaller molecular weights of chitosan in the liquid solution could pass the bacterial cell membrane and bind with DNA.[10] One of the most prevalent biominerals, calcium carbonate ($CaCO_3$), has garnered interest in numerous studies. The CaCO3 nanoparticles (CaCO3-NPs) possess considerably enhanced features and functionalities, so their architecture has really become vital for biotechnological and industrial applications as results of the unique characteristics of CaCO3 nanomaterials, such as simple synthesis, large surface-to-volume ratio, robust character and surface functionalization, and capability to occur in a diverse of polymorphs and shapes[14] Al-Ataees *et al*.[15] showed that $CaCO_3$-NPs present as a superior antibacterial agent on G+ and G− bacteria such as *Agrobacterium tumefaciens* and *S. aureus*. $CaCO_3$-NPs could also provide advantages in enhancing the intracellular penetration of antibiotics and retention time to achieve its efficacy, and thus, it can be presented as potential antimicrobial agent delivery system. The current study aimed to isolate and identify *S. aureus*, *S. pyogenes*, *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* from various clinical sources and study the antibiotic resistance patterns. In addition to evaluate the antibacterial effect of two types of nanoparticles (CS-NPs and CaCO3-NP) at various concentration on isolated bacteria *in vitro*.

Materials and Methods

Bacterial samples collection and diagnosis

Overall 128 clinical samples (including sputum, blood, urine, and ear) were collected from out- and inpatients who were referred to Al-Hilla Teaching Hospital for 3 months (from 2023 July to September 2023). These samples were rapidly seeded on blood agar media and MacConkey agar media, and subsequently put in an incubator at 37°C for 24 h in aerobic enviroments. All isolates of bacteria involving *S. aureus*, *Streptococcus pyogenes*, *S. pneumoniae*, *P. aeruginosa*, *E. coli*, and *K. pnemoniae* were isolated, determined based on their diagnostic features, and then checked with the data provided in standard references.[16] The isolates were confirmatory identified by the VITEK2 system by utilizing a VITEK2 GN kit, then kept on maintaining medium for additional testing.

Antimicrobial susceptibility testing

The National Committee for Clinical Laboratory Standards' recommendations were followed for testing the antibiotic sensitivity on Mueller-Hinton agar medium by using Kirby-Bauer disk diffusion test.[17] The antibiotic disks (Bioanalyse, UK; Merck kGaA; and Sigma-Aldrich) used are ceftazidime (CAZ), gentamicin (GN), amikacin, trimethoprim (TMP), nitrofurantoin (F), ciprofloxacin (CIP), levofloxacin, and nalidixic acid (NA).

Preparation of chitosan nanoparticle solution

Nanochitosan particles (85% deacetylated) were supplemented by Sigma-Aldrich (Germany). N-trimethyl chitosan was bought from the HeppeMedical Chitosan GmbH. Heinrich-Damerow-Straße Halle (Saale), Germany. Sodium hydroxide and pentasodium triphosphate ($Na_5P_3O_{10}$) were supplemented from Merck kGaA (Germany). In an aqueous 10% acetic acid solution, 10% chitosan was dissolved, and the mixture was mixed at 400 rpm overnight at room temperature. After the pH was regulated to 4.6 with persistent mixing for about 45 min, the mixture was combined with (1%) thymol and shaken for 2 h at room temperature by adding a few drops of tripolyphosphate (0.10 %) solutions. The resultant mixture was stirred, centrifuged in a round (15,000 rpm) about 15 min, rinsed numerous times with distilled water, and then sonicated for 3 h at 25°C. The preparation of chitosan solutions is as follows: chitosan solution at concentrations of 20, 40, 60, and 80 mg/mL was made via combining 0.020, 0.040, 0.060, and 0.080 g sequentially of chitosan with 100 mL of 2% v/v acetic acid and distilled water.

Preparation of $CaCO_3$ nanoemulsion

$CaCO_3$-NPs (99.95%) with an approximate size of about 20 nm were provided by Sigma-Aldrich (St. Louis, Missouri). The recommended emulsifier for the $CaCO_3$ emulsion used in medicinal uses is a sugar fatty acid ester. The primary particles have a mean particle diameter between 7 and 9 nm, whereas the secondary particles as a whole have an average particle diameter between 11 and 14 nm. To keep a suitable dispersing state, a spectrum of $CaCO_3$ particles (5%–20% by weight) and an emulsifier (0.1%–5% by weight) are distributed in the water.

We may obtain a $CaCO_3$ emulsion that is simple to make and simple to use, then use various concentrations of

nanoemulsion (20, 40, and 60 mg of $CaCO_3$ in 80, 60, and 40 mL of combined water and vinegar [CH3COOH]) equally.

Based on the interaction:

$$CaCO_3 + 2CH_3COOH \rightarrow Ca(CH_3COO)_2 + H_2O + CO_2$$

the concentration will have to be:

20/(20 + 80) = 20%

40/(40 + 60) = 40%

60/(60 + 40) = 60%

80/(80 + 20) = 80%

Screening for antibacterial activity

Antimicrobial activities of various (CS-NPs and $CaCO_3$-NPs) concentrations (20, 40, 60, and 80 mg/L) were tested *in vitro* against bacterial isolates by the agar diffusion technique, sterile swab immersed into the broth of microorganisms with a 24-h-old culture of the bacteria were used to culturing Mueller-Hinton agar plates. The inoculated amount was adjusted to produce a concentration of about 1.5 × 108 colony-forming units per milliliter, especially in comparison to the turbidity of 0.5 McFarland standards. Five wells (5 mm) were then formed in the agar using a cork borer, and each well received 0.1 mL of every nanoparticle concentration from both solutions mixed in (dimethyl sulfoxide) that had been made previously in varied concentrations. The control solution (distilled water) was placed in the central well. To ensure proper diffusion, the plates were first let to incubate at 5–8°C for about 2–3 h and further put in an incubator at 37°C overnight. The inhibiting area diameter (mm) produced around the well was used to assess the antibacterial effectiveness. Standard disc of gentamycin (10 mcg/ disc) was used as a positive control for antibacterial activity.[17,18]

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the samples were taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee, according to the document number 45 (including the number and the date on June 3, 2022) to get this approval.

Results

Bacterial isolation and characterization

In these investigations, mainly selected bacteria such as *S. aureus*, *S. pyogenes*, *K. pneumoniae*, *E. coli*, and *P. aeruginosa* were obtained from various clinical specimens such as urine, wound, blood, sputum, and ear. The most frequently isolated bacteria have been *P. aeruginosa* (37.9%) followed by *E. coli* (31.2%), *S. aureus* (20%), *S. pyogenes* (7.5%), and *K. pneumoniae* (3.7%) [Table 1]. The most isolated pathogens from the wound and urine were *P. aeruginosa and E. coli*, respectively. The most frequently used pathogen for blood infection was *S. aureus*.

Antimicrobial activity test

Isolated bacteria were screened for their sensitivity to several antimicrobials to determine their antimicrobial susceptibility. In general, most isolates showed elevated resistance to most antibiotics, and mainly, bacterium isolates exhibited minimal levels of fluoroquinolone resistance. It was found that CAZ was the least effective antibiotic against most of the bacteria [Figure 1]. In the present study, *P. aeruginosa* exhibited relatively high resistance to most antibiotics (100%) CAZ, GN, TMP, F, and NA.

Determination of the antimicrobial activity nanoparticles by agar diffusion method

Chitosan nanoparticles

The findings of this investigation have demonstrated the antibacterial effects of nanochitosan and nano-$CaCO_3$, which may be significant in limiting bacterial infections in patients because these nanomaterials seem to be acting as therapeutic agents that treat infectious diseases. In the current study, the two kinds of nanosubstance impacts toward various G+ and G− bacteria gained from patients were tested. Antimicrobial activity of CS-NPs was investigated by well diffusion test versus five bacteria: *S. aureus*, *S. pyogenes*, *P. aureginosa*, *E. coli*, and *K. pnemoniae*. The results of zone inhibition methods have been described in Table 2; it is seen that different prepared concentrations ranging from 20 to 80 mg/L of CS-NPs show good inhibition zone and

Table 1: Frequency of bacterial types isolated from different cases

Samples	No. of positive samples (%)	*S. aureus*	*S. pyogenes*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
Wound (66)	43 (65%)	6 (13.9%)	1 (2.3%)	9 (20.9%)	2 (4.6%)	25 (58%)
Blood (6)	5 (83%)	3 (60%)	0	1 (20%)	0	1 (20%)
Ear (7)	7 (100%)	2 (28.5%)	3 (42.8%)	1 (14.2%)	0	1 (14.2%)
Urine (44)	22 (50%)	4 (18.1%)	0	14 (63.6%)	1 (4.5%)	3 (13.6%)
Sputum (5)	3 (60%)	1 (33.3%)	2 (66.6%)	0	0	0
Total (128)	80 (62.5%)	16 (20%)	6 (7.5%)	25 (31.2%)	3 (3.7%)	30 (37.9%)

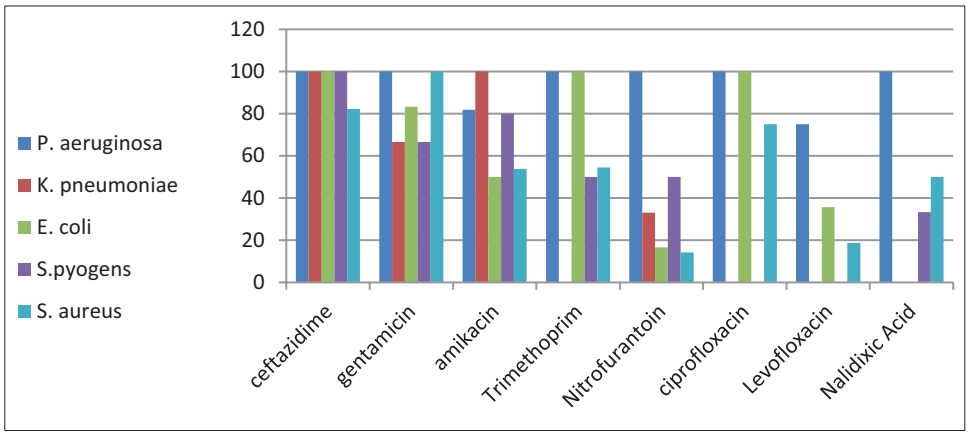

Figure 1: The resistant rate of bacteria to the antibiotics

Table 2: The inhibitory antibacterial effect (inhibition zone mm) of different Chitosan nanoparticle concentrations against different bacteria

Bacteria	Chitosan concentration mg/L				GN
	20	40	60	80	10 mcg
S. aureus	17	21	23	25	12
S. pyogenes	23	23	24	24	14
E. coli	14	14	15	20	20
P. aeuroginosa	21	22	23	24	16
K. pnemoniae	23	24	25	27	17

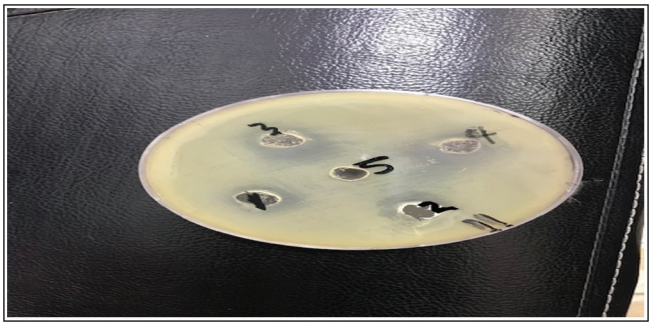

Figure 2: The impacts of various nano-chitosan concentrations against *E. coli*: (1) 20 mg/mL; (2) 40 mg/mL; (3) 60 mg/mL; (4) control (D.W)

repressed the amplifying of all bacteria. As the concentration of CS-NPs increased, the antibacterial levels increase and showed the highest inhibition zone at the concentration of 80 mg/L. Figures 2 and 3 show the efficacy of nano-CS on *E. coli.* and *S. aureus*, respectively.

CaCO3 nanoparticles

Bacterial isolates were exposed to different concentrations of $CaCO_3$-NPs, and it is noticed inhibition of all examined bacteria. Different sizes of inhibition zone depend on the type of pathogenic bacteria, in which *E. coli* revealed the lowest inhibition zone at the concentration of 80 mg/L, as

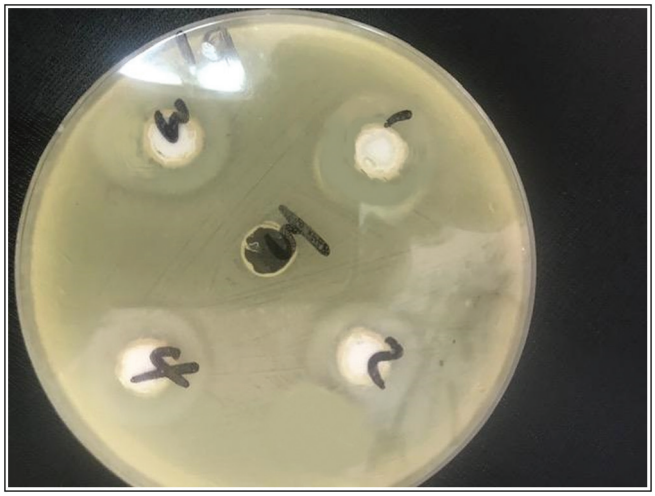

Figure 3: The impact of various nano-chitosan concentrations against *S. aureus*: (1) 20 mg/mL, (2) 40 mg/mL, (3) 60 mg/mL, (4) 80 mg/mL, (5) control (D.W)

Table 3: The inhibitory antibacterial effect (inhibition zone mm) of different $CaCO_3$ nanoparticle concentrations against different bacteria

Bacteria	$CaCo_3$ concentration mg/L				GN
	20	40	60	80	10 mcg
S. aureus	20	21	23	25	12
S. pyogenes	20	25	27	30	14
E. coli	20	21	22	24	20
P. aeuroginosa	17	19	20	23	16
K. pnemoniae	17	19	21	24	17

shown in Table 3. The antimicrobial effects were shown to be dose-dependent and increase as the concentration increased. At the concentration of 80 mg/L, nanoparticle showed the largest inhibition zone against *S. pyogenes* (40 mm). Figures 4 and 5 show the activity against *S. aureus* and *P. aeruginosa.*

Figure 4: The impact of various nanocalcium carbonate concentrations against *S. aureus*: (1) 20 mg/mL, (2) 40 mg/mL, (3) 60 mg/mL, (4) 80 mg/mL, (5) control (D.W)

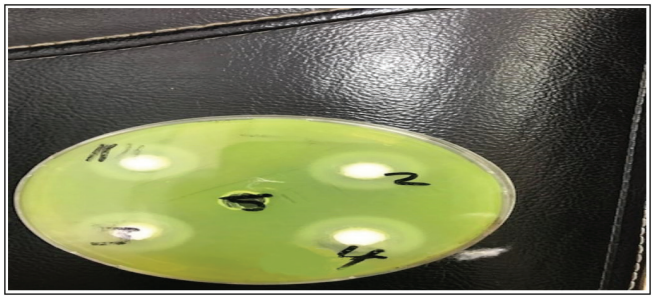

Figure 5: The impact of various nanocalcium carbonate concentrations s against *S. aureus*: (1) 20 mg/mL, (2) 40 mg/mL, (3) 60 mg/mL, (4) 80 mg/mL, (5):control (D.W)

Discussion

The present study showed that *P. aeruginosa* seems to be the common microorganism; it was detected with a remarkable frequency, in which it is commonly isolated from the wound and urine. Similar results have been reported by Hassanzaden *et al*.[19] that *P. aeruginosa* is the most common cause of bacterial infection (39.1%). Other studies[20] stated that *P. aeruginosa* is the most commonly isolated bacteria. In contrast, Mulu *et al*.[21] mentioned that S. aureus was most common predominant isolated from various infections (31.5%) followed by *E. coli* (13.8%) and *P. aeruginosa* 30 (10.3%). According to previous studies.[22], Klebsiella was the most often isolated pathogen, followed by *E. coli*, *P. aeruginosa*, and *S. aureus*.[22] *P. aeruginosa* is an opportunistic bacterium that is widely dispersed and causes a variety of infections in hospitals and the community, especially in people with weakened immune systems. It is extremely difficult to control *P. aeruginosa* in a hospital environment because of the organism's wide habitat and range, which makes it difficult to manage contamination.[23]

The isolated bacteria exhibited remarkable resistance to the majority of antimicrobial agents, and this may be due to the widespread use of these therapeutics leading to the emergence of resistance. *Pseudomonas* revealed resistance against most antibiotics. Previous investigations like,[24] registered worrying elevated levels of antimicrobial resistance in which, *P. aeruginosa* bacteria displayed high unsusceptibility to mainly antibiotics, CIP and NA appeared to be the most effective antimicrobials against the studied microorganisms.

P. aeruginosa is inherently resistant to several antimicrobials, and antibiotic resistance factors can be easy to transfer. Additionally, *P. aeruginosa* is highly likely to develop multidrug resistance.[25] Multiple resistance strategies significantly affect clinical outcomes because they reduce the therapeutic treatment strategies for *P. aeruginosa* pathogens, limit the effectiveness of antipseudomonal medicines, and make infection with exceedingly challenging to cure.[26] Mulu *et al*.[21] demonstrated that *P. aeruginosa*, *E. coli*, and *S. aureus* had significant levels of GN resistance and low levels of CIP resistance. It reported that microbes possess approximately low resistance level to nitrofurantoin and high prevalence of resistance to CAZ, but *E. coli* exhibited a greater degree of resistance amid overall microorganisms than *Klebsiella, Staphylococcus*, and *Pseudomonas*.[27] Multidrug-resistant bacteria are very common, as results of excessive utilization of medication that are commonly described for combat diseases,[28] which puts pressure on bacteria to develop resistance. Ineffective antibiotics provided by doctors and poor patient compliance with antibiotic programs could also result in gene mutation and resistance.[29]

The antibacterial activity of nanochitosan has been investigated by the disk diffusion method toward isolating bacteria. CS-NPs revealed promising antimicrobial properties toward pathogenic bacteria. The inhibitory action of CS-NPs varied depending on the kind of bacteria. These findings concur with earlier research that CS-NPs displayed remarkable antibacterial efficacy on pathogens that are significant to medicine including *S. pyogenes*, *S. aureus*, *Salmonella typhi*, *P. aeruginosa*, and *E. coli*, whereas the *Enterococcus faecalis* seemed to be a resistant strain.[28] The author[30] determined that CS-NPs exhibited high inhibition rates against the investigated pathogenic microbes (*P. aeruginosa*, *E. coli*, *K. pneumonia*, and *S. aureus*) and that the effectiveness enhanced as the concentration of CS-NP concentration increased. Numerous studies have reported on the widespread antimicrobial property of chitosan versus bacteria and fungi. Furthermore, the efficacy of the antimicrobial action of chitosan is significantly based on the kind of microorganism.[31] CS-NPs exhibit higher antimicrobial action due to the enhanced ratio of volume to surface of nanomaterials.[32] CS-NPs can offer considerable antibacterial characteristics through a number of routes. These involved the reaction between CS-NPs (positive charge) and phospholipids (negative charge) of the plasma membrane, the ability of CS-NPs to chelate, their capacity to pass cell walls, and their capability to impede DNA and mRNA syntheses.[33]

$CaCO_3$ nanoparticles have antimicrobial action toward various isolated bacteria. The inhibiting level was directly

associated with the concentration of $CaCO_3$-NPs. The different examined bacterial species had different responses to the nanoparticles that were evaluated. These findings are in agreement with previous reports that $CaCO_3$-NPs showed the influence on bacterial growth, including: *S. pyogenes*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *Serratia marcescens*. The results of the experiment indicated that smaller NP sizes have higher antibacterial effects.[34] The study carried out by Rinun *et al*.[35] showed that the $CaCO_3$-NPs possess highly antibacterial effect on *E. faecalis*, *Bacillus subtilis*, *E. Coli*, and *P. aeruginosa*. The lack of growth surrounding the nanomaterial is an indirect indicator of the capability of the substances to reduce the growth. $CaCO_3$-NP is capable to bind to the biological molecules, which confers greater efficiency. It is proposed that it may affect all membranes of the bacteria and so prevent their growth. It was observed that nanostructures interact with the cytoplasmic membrane and cell walls of *E.coli* causing gaps in the bacterial cell wall and eventually killing it.[36]

Conclusion

Bacterial pathogens are major health problems, and many bacterial diseases are associated with elevated resistance levels; the study is a good demonstration that CS-NPs and $CaCO_3$-NP exhibited good activity against the tested bacteria. It can be applied as an effective antimicrobial and seem to have a beneficial impact on microbial reduction; therefore, it is suitable for the treatment of infections by investigating bacteria. In light of the previous findings, these nanoparticles are recommended as a potential candidate for the development of antimicrobial medications utilized in numerous domain areas of medicine, agriculture and food industry, and agriculture.

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Conflicts of interest

There are no conflicts of interest.

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