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Research Article/Araştırma Makalesi

In Vitro Antibacterial Activity of Chitosan/ZnO/Ag Ternary Nanocomposites against Clinical Wound Isolates

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Abstract: Chitosan is a biodegradable, linear, biocompatible natural polymer. By adding silver (Ag) and zinc oxide (ZnO) nanoparticles to a gel, a bandage with antibacterial, antimicrobial, and anti-inflammatory effects can be formed. In this study, the antibacterial efficacy of pure chitosan nanoparticles and Cs/ZnO/Ag nanocomposite solutions against both Gram-positive and Gram-negative bacteria was investigated. *Staphylococcus aureus* was the most frequently detected bacterium at 16%. *Klebsiella pneumoniae* ranked second at 11%, and *Escherichia coli* ranked third at 5%. The inhibition zones in millimeters around agar wells containing pure chitosan nanoparticle solutions and Cs/ZnO/Ag nanocomposite solutions were measured in this study. The inhibition zones against *S. aureus* were 5, 8 and 10 mm respectively for pure solutions of chitosan nanoparticles and 5, 7 and 10 mm respectively for *E. coli*. Similarly, the inhibition zones for *K. pneumoniae* were 5, 8, and 10 mm, respectively. The Cs/ZnO/Ag nanocomposites were tested in solution at different ZnO:Ag ratios. It was observed that the antibacterial activity increased when CS/ZnO/Ag nanocomposites were applied at different ratios (ZnO:Ag = 1:1, 2:1, and 3:1; 0.15% by weight). In this group, inhibition zones for *S. aureus* were measured as 9, 9, and 10 mm; for *E. coli* as 8, 10, and 10 mm; and for *K. pneumoniae* as 9, 10, and 11 mm. In conclusion, enriching chitosan-based nanocomposites with ZnO and silver nanoparticles significantly increases their antibacterial efficacy against both Gram-positive and Gram-negative bacteria. These findings suggest that CS/ZnO/Ag nanocomposites can be used as a potential biomaterial in the control of wound infections.

Keywords: Chitosan, zinc oxide (zno), silver (ag), nanoparticles.

Kitosan/ZnO/Ag Nanokompozitlerin Klinik Yara İzolatlarına Karşı İn Vitro Antibakteriyel Aktivitesi

Öz: Kitosan; biyolojik olarak parçalanabilir olması, doğrusal yapısı ve biyouyumlu olması gibi pek çok olumlu özelliğe sahip doğal bir polimerdir. Gümüş (Ag) ve çinko oksit (ZnO) nanopartiküllerinin bir jele dâhil edilmesiyle; antibakteriyel, antimikrobiyal ve antienflamatuvar etkiye sahip bandaj oluşturulabilmektedir. Bu çalışmada, saf kitosan nanopartiküllerinin ve Cs/ZnO/Ag nanokompozit çözeltilerinin hem Gram-pozitif hem de Gram-negatif bakterilere karşı antibakteriyel etkinliği araştırılmıştır. En sık tespit edilen bakteri %16 ile *Staphylococcus aureus* olmuştur. *Klebsiella pneumoniae* %11 ile ikinci, *Escherichia coli* ise %5 ile üçüncü sırada yer almıştır. Bu çalışmada, saf kitosan nanopartikül çözeltileri ve Cs/ZnO/Ag nanokompozit çözeltileri içeren agar kuyucuklarının etrafında oluşan hale şeklindeki inhibisyon bölgeleri milimetre cinsinden ölçülmüştür. *S. aureus*'a karşı inhibisyon bölgeleri, saf kitosan nanopartikül çözeltileri için sırasıyla 5, 8 ve 10 mm; *E. coli* için ise sırasıyla 5, 7 ve 10 mm olarak belirlenmiştir. Benzer şekilde, *K. pneumoniae* için inhibisyon bölgeleri de sırasıyla 5, 8 ve 10 mm olarak ölçülmüştür. CS/ZnO/Ag nanokompozitlerinin farklı oranlarda (ZnO:Ag = 1:1, 2:1 ve 3:1; %0.15 ağırlıkça) uygulanması sonucunda antibakteriyel etkinliğin arttığı gözlenmiştir. Bu grupta *S. aureus* için inhibisyon zonları 9, 9 ve 10 mm; *E. coli* için 8, 10 ve 10 mm; *K. pneumoniae* için ise 9, 10 ve 11 mm olarak ölçülmüştür. Sonuç olarak, kitosan bazlı nanokompozitlerin ZnO ve gümüş nanoparçacıkları ile zenginleştirilmesi, hem Gram-pozitif hem de Gram-negatif bakterilere karşı antibakteriyel etkinliği anlamlı düzeyde artırmaktadır. Bu bulgular, CS/ZnO/Ag nanokompozitlerinin yara enfeksiyonlarının kontrolünde potansiyel bir biyomalzeme olarak kullanılabileceğini göstermektedir.

Anahtar kelimeler: Kitosan, çinko oksit (zno), gümüş (ag), nanoparçacıklar.

1. Introduction

Nanocomposite technology has received much attention for several biomedical applications such as drug delivery, tissue engineering, wound healing, and thermal therapy. Numerous aspects of the nanocomposite, including its mechanical and thermal qualities, antibacterial qualities, balance and structure with porosity to enable absorption, and capacity to give the ideal humidity in the wound bandage, make it suitable for the wound healing process (Shabunin et al., 2019). Because of their availability, special qualities, and biodegradability, biopolymers made from sustainable resources are a major topic of attention in this technology (Fatullayeva et al., 2022). The possibility of producing useful materials has led to a great deal of research on polysaccharides, one of the several types of biopolymers. Cellulose, chitin, and starch particularly are frequently researched for their potential uses in a variety

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of industries, such as the food, pharmaceutical, and medical sectors (Zhang & Wang, 2015). Because polysaccharides are biocompatible and biodegradable, they are perfect for tissue engineering, wound healing, and drug delivery systems (Wang et al., 2016).

The distinctive characteristics of chitosan (CS), the second-largest renewable biopolymer after cellulose, including its antibacterial activity, nontoxicity, and biocompatibility, have garnered significant interest in a number of areas (Saeedi et al., 2021). Its prospective uses are, however, limited by its weak electrical and mechanical characteristics (Saravanan et al., 2014). Hence, adding nanofillers is a practical way to enhance the mechanical and physical characteristics of chitosan (Yadav et al., 2014). Furthermore, chitosan is a great adsorbent for heavy metal ions and dye absorption due to its abundance of amino and hydroxyl groups (Li et al., 2020). Recently, nanoparticles have shown promise as additions to enhance the characteristics of biopolymers. Because of its broad-spectrum antibacterial properties, biocompatibility, and affordability of manufacture, zinc oxide nanoparticles, or ZnO NPs, are utilized extensively in many different industries (Bailore et al., 2021). According to several reports, chitosan-coated ZnO nanocomposites are an essential component for a number of uses, including dye removal, textile coating, and dietary production (Asadzadeh Patehkhor et al., 2021; Ngamsurach et al., 2022), based on their antimicrobial (Rajabloo et al., 2022), antibiofouling, and photocatalytic activity (Mostafa et al., 2020). Metal-based nanoparticles (NPs) such as Ag, ZnO, TiO, Cu, CuO, and Au have demonstrated exceptional antibacterial effectiveness against antibiotics, especially when compared to antibiotic-resistant bacteria. These metal nanoparticles produce reactive oxygen species (ROS), which damage microbial DNA and interact with the microbe's proteins and organic components to generate electrolyte imbalance, which ultimately kills the microorganisms (Sklodowski et al., 2023). For example, enterotoxigenic *E. coli* (ETEC) cannot adhere to enterocytes or internalize them when zinc oxide (ZnO) is present (Yadi et al., 2018). Furthermore, ZnO nanoparticles (ZnO-NPs) have antibacterial properties and can lessen microbial adhesion and survival on biomedical surfaces (Salem et al., 2015). Remarkably, a number of findings point to ZnO-NPs' selective toxicity, which mostly affects prokaryotic systems, yet it has also been shown to kill cancer cells (Shanmugasundaram & Balagurunathan, 2017). Additionally, a number of microbes are extremely poisoned by Ag+ ions and Ag-based compounds, which makes them intriguing candidates for a variety of medicinal applications (Ahmad et al., 2024). Although silver is often utilized as a nitrate salt, its antibacterial activity is significantly augmented when it is present in the form of Ag nanoparticles (Ag-NPs), which have a larger surface area (Crisan et al., 2021).

2. Material and Method

2.1. Sample Collection

A total of 150 wound swab specimens were collected from patients with wound infection admitted in Al-Najaf Al-Ashraf city, Iraq, including Al-Hakeem General Hospital and Al-Zahraa Teaching Hospital. The study was conducted from August 2024 to January 2025 at Al-Hakeem General Hospital. Samples were collected by aseptic means with sterile swabs and transferred immediately into sterile transport medium. The swabs were transported to the microbiology laboratory for further analysis. Samples not processed immediately were stored at 4°C for short-term storage. No storage of the original wound swab samples was performed for long term; only purified bacterial isolates were stored under appropriate laboratory conditions for subsequent analyses. (Haalboom et al., 2018)

2.2. Sample Cultivation

Sterilized swabs were used to collect all samples. The manufacturer's instructions were followed in the preparation of the culture media, which included three different types: MacConkey, Blood Agar, and Sabouraud dextrose agar. These three culture media were used to cultivate the samples. Following 24 h of incubation, the samples were evaluated for bacterial identification and antimicrobial susceptibility testing using the VITEK 2 automated system (bioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions as previously evaluated for clinically relevant bacteria by Ligozzi et al. (2002).

2.3. Preparation of Chitosan NPs/Acetic Acid Solution

Chitosan powder (BDH, England) was weighed by sensitive electronic balance (Sartorius, Germany) to prepare the CS/acetic acid solutions. Chitosan/acetic acid solutions were prepared by dissolving accurately weighed chitosan powder (BDH, England) into 100 mL of diluted acetic acid solution (acetic acid:distilled water, 1:10, v/v; Sigma-Aldrich/MilliporeSigma, USA). Three solutions of viscous chitosan with concentrations of 0.25, 0.5 and 1.0 g/mL were prepared under continuous stirring until complete dissolution. The utilization of diluted acetic acid as a solvent for chitosan is based on the fact that chitosan is soluble in acidic media due to the protonation of amino groups. This method is similar to the common method described by Qi et al. (2004) where chitosan was dissolved in acetic acid prior to nanoparticles preparation by ionic gelation with tripolyphosphate. However, the concentrations of chitosan and the ratio of acetic acid used in the present study have been modified. (Qi et al., 2004)

2.4. Preparation of Cs/ZnO and Cs/Ag Nanocomposites

The nanocomposites were generated by loading chitosan (CS) with varied weight ratios of zinc oxide nanoparticles (ZnO NPs; particle size 10–50 nm) and silver nanoparticles (Ag NPs; particle size 10–30 nm) at 0.05%, 0.10% and, 0.15% w/w. The mixtures were sonicated for 12 min with a pulse mode of 5 s on/5 s off at 25% amplitude/power. The samples were sonicated in an ice bath to avoid overheating and to ensure that nanoparticles were uniformly dispersed in the CS matrix. The synthesis of metal oxide- or metallic nanoparticle-based chitosan nanocomposites is carried out by dispersing nanoparticles within the polymer matrix via sonication to improve homogeneity and reduce aggregation (Souza et al., 2020; Premanathan et al., 2011).

2.5. Antibacterial Effects of CS/ZnO/Ag Nanocomposites

The produced nanocomposite solutions' antibacterial properties were evaluated against Gram-positive and Gram-negative bacteria using *S. aureus*, *E. coli*, and *K.*

pneumoniae, respectively. Prior to the experimental procedure, the used Petri dishes were autoclaved. Five or six distinct colonies were spread on Petri plates and incubated for twenty-four hours at 37°C in order to prepare the medium for bacterial culture. The medium was produced and inoculated with three distinct kinds of bacteria. To test nanocomposites, a large number of wells (6 mm in diameter) were made in the medium and the nanocomposites were then applied to the wells utilizing a micropipette (10 µL) (Govindasamy et al., 2021).

2.6. Statistical Analysis

Statistical analysis was conducted on the values of the inhibition zones obtained from the mean of triplicate measurements. The reported standard deviation (SD) values reflect inter-species variability across the three bacterial species tested (*S. aureus, E. coli,* and *K. pneumoniae*) at each treatment level, rather than intra-species technical replicate variation. Descriptive statistics were used to summarize inhibition zone diameters as mean ± standard deviation. Given the small per-group sample size (n = 3 bacterial species per treatment level), the assumptions of normality and homogeneity of variance required for parametric testing could not be verified; therefore, one-way ANOVA was not applied. Instead, non-parametric tests were used throughout. The relationship between treatment level and inhibition zone diameter was assessed using Spearman correlation analysis; noting that with perfectly monotone data (n = 3) a Spearman coefficient of r = 1.0 is mathematically inevitable and should be interpreted as a descriptive trend indicator rather than an inferential result. The antibacterial performance of pure CS nanoparticles and CS/ZnO/Ag nanocomposites was compared using the Mann–Whitney U test and effect size was calculated using Cohen's d. Kruskal–Wallis analysis was used to compare the responses of different bacterial species within the nanocomposite group; however, given the limited per-species sample size (n = 3), these results should be regarded as exploratory. Statistical significance was accepted at $p < 0.05$. (Cohen, 2013; Chicco et al., 2025)

3. Results and Discussion

A total of 150 wound specimens were collected from 112 patients who were hospitalized to various hospitals in Al-Najaf Al-Ashraf city (Al-Hakeem General Hospital and Al-Zahraa Teaching Hospital). Of these patients, 60 (53%) were female, whose ages varied from 20 to 80 years. There were 28 distinct microbial species identified in all, of which 44.2% were Gram-positive and 55.8% were Gram-negative. *K. pneumoniae* (11%), *E. coli* (5%), and *S. aureus* (16%) were the most frequently found bacterial species as can be seen in Figure 1.

The isolation results showed that most of the wounds were infected with one species of bacteria. In the present study the most frequently isolated bacterium was *Staphylococcus aureus*. This finding is consistent with the previous reports in which *S. aureus* was one of the predominant bacterial species isolated from different types of wound infections and was responsible for approximately 40–60% of bacterial isolates in some studies (Puca et al., 2021). According to the previous investigations, *P. aeruginosa* was the Gram-negative that was most frequently found (Wood et al., 2023). The results of the inhibition zone growth of *S. aureus* bacteria are displayed in Figure 2 and Table 1 following treatment with CS NPs at varying concentrations (0.25 g, 0.5 g, and 1.0 g), with inhibition zones (IZ) of 5, 8, and 10 mm, respectively. Inhibition zones (IZ) for *E. coli* were 5, 7, and 10 mm, in that order. In contrast, *K. pneumoniae* had inhibition zones (IZ) of 5, 8, and 10 mm.

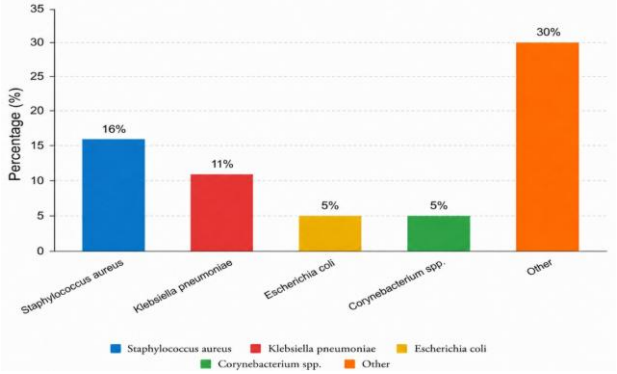

Figure 1. Percentage distribution of microorganisms isolated from 150 wound swab samples collected from infected wound patients. *Staphylococcus aureus* represented 16% of the isolates, *Klebsiella pneumoniae* 11%, *Escherichia coli* 5%, *Corynebacterium* spp. 5%, and other microorganisms 30%.

In previous research, chitosan nanoparticles were shown to be able to stop the development of bacteria at high concentrations (Ali & Aldujaili, 2022). The findings demonstrated that both Gram-positive and Gram-negative bacteria might be inhibited by nanoparticles. Gram-positive bacteria were more sensitive to biogenic chitosan nanoparticles than Gram-negative bacteria (Ali et al., 2022). This is consistent with the finding that Gram-positive bacteria are more vulnerable to the antibacterial effects of chitosan than Gram-negative bacteria (Ke et al., 2021). Consequently, it is challenging to evaluate the bacteria's interaction with chitosan.

The scientific field has designated it their goal to discover an efficient alternative medication complement to antibiotics in order to substitute for the current antibiotics that have developed resistance in places due to the growing concern of multidrug resistance (MDR). Nowadays, nanoparticles are viewed as a viable alternative to antibiotics and appear to have significant potential for solving the MDR problem caused by bacteria (Yang et al., 2021). Following treatment with CS/ZnO/Ag NPs in varying ratios at higher ZnO and Ag NPs concentrations (0.15 wt%), ZnO/Ag ratios of 1:1, 2:1, and 3:1 were used for ZnO nanoparticles. When *Staphylococcus aureus* bacteria were tested, the results revealed inhibition zone growth of 9, 9, and 10 mm, respectively. Inhibition zone growth for *E. coli* was 8, 10, and 10 mm. As shown in Table 2 and Figure 3, *K. pneumoniae* exhibited inhibitory zone development of 9, 10, and 11 mm.

Descriptive analysis of the inhibition zone data showed a clear concentration-dependent increase in the antibacterial activity of pure CS nanoparticles. Across the three bacterial species tested, the mean inhibition zone increased from 5.00 ± 0.00 mm at 0.25 g to 7.67 ± 0.58 mm at 0.5 g and 10.00 ± 0.00 mm at 1.0 g. This trend suggested that increasing CS concentration enhanced antibacterial activity against both Gram-positive and Gram-negative bacteria.

Table 1: Antibacterial activities of CS NPs at different concentrations against *Staphylococcus aureus*, *E. coli*, and *Klebsiella pneumoniae* (inhibition zone in mm).

CS Concentration	*Staphylococcus aureus* IZ (mm)	*E. coli* IZ (mm)	*Klebsiella pneumoniae* IZ (mm)	Mean ± SD (mm)
0.25 g	5	5	5	5.00 ± 0.00
0.5 g	8	7	8	7.67 ± 0.58
1.0 g	10	10	10	10.00 ± 0.00

Table 2: Antibacterial activities of CS/ZnO/Ag NPs with different ZnO:Ag ratios (1:1, 2:1, and 3:1) at 0.15% w/w concentration, showing inhibition zone growth against *Staphylococcus aureus*, *E. coli*, and *Klebsiella pneumoniae* (mm).

ZnO/Ag Ratio	*Staphylococcus aureus* IZ (mm)	*E. coli* IZ (mm)	*Klebsiella pneumoniae* IZ (mm)	Mean ± SD (mm)
1:1	9	8	9	8.67 ± 0.58
2:1	9	10	10	9.67 ± 0.58
3:1	10	10	11	10.33 ± 0.58

Table 3: Descriptive statistical summary of inhibition zone results. SD values represent inter-species variability across the three bacterial species tested (n = 3) at each treatment level.

Group	Treatment Level	Mean IZ ± SD	Interpretation
CS NPs	0.25 g	5.00 ± 0.00 mm	Lowest activity
CS NPs	0.5 g	7.67 ± 0.58 mm	Moderate activity
CS NPs	1.0 g	10.00 ± 0.00 mm	Highest activity
CS/ZnO/Ag	1:1	8.67 ± 0.58 mm	Lower nanocomposite activity
CS/ZnO/Ag	2:1	9.67 ± 0.58 mm	Increased activity
CS/ZnO/Ag	3:1	10.33 ± 0.58 mm	Highest nanocomposite activity
CS NPs vs CS/ZnO/Ag	Overall	7.56 ± 2.19 vs 9.56 ± 0.88 mm	Higher in nanocomposite; Cohen's d = 1.20

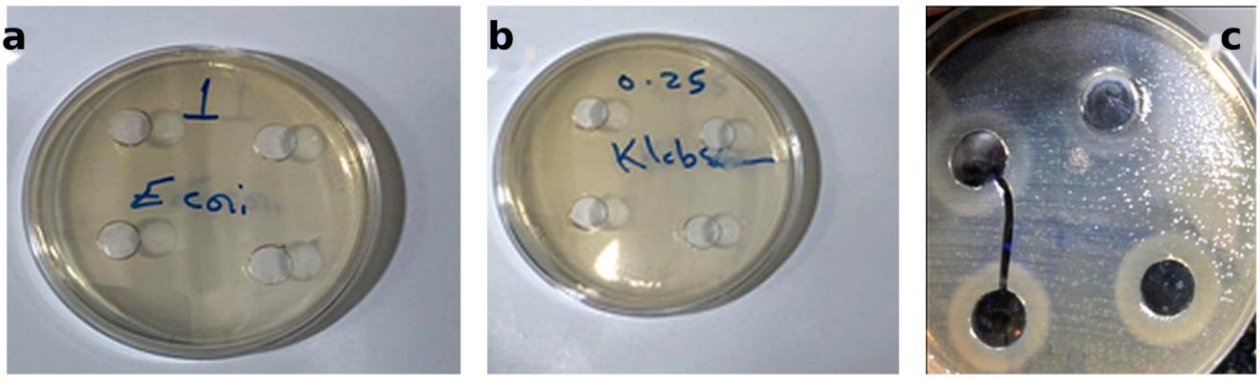

Figure 2. A: Growth of *E. coli*; B: Growth of *K. pneumoniae*; C: Growth of *S. aureus* treated with CS NPs at different concentrations.

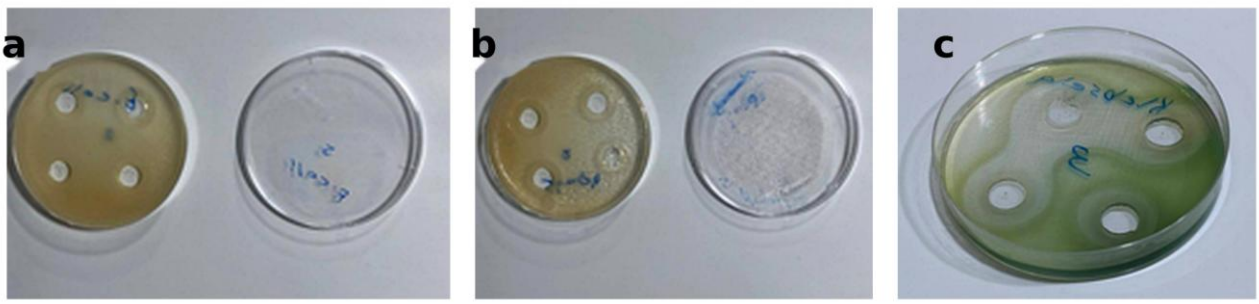

Figure 3. A: Growth of *E. coli*; B: Growth of *Staphylococcus aureus*; C: Growth of *Klebsiella pneumoniae* treated with CS NPs mixed with ZnO/Ag NPs at a 3:1 ratio.

The same increasing trend was also observed in the group of CS/ZnO/Ag nanocomposite with increased concentration of ZnO:Ag. The mean inhibition zones were 8.67 ± 0.58 mm for the 1:1 ratio, 9.67 ± 0.58 mm for the 2:1 ratio and 10.33 ± 0.58 mm for the 3:1 ratio. These results demonstrated that the incorporation of ZnO and Ag nanoparticles improved the antibacterial performance of chitosan and the 3:1 ZnO:Ag formulation exhibited the highest overall inhibition zone among the tested ratios.

The CS/ZnO/Ag nanocomposite group had a higher mean inhibition zone than pure CS nanoparticles considering all bacterial species and treatment levels together (9.56 ± 0.88 mm vs. 7.56 ± 2.19 mm, respectively). Note that these SD values represent inter-species variability (n = 3 bacterial species), not technical replicate variation; the larger SD in the CS NPs group (2.19 mm) reflects the wider spread of inhibition zones across concentrations, while the smaller SD in the CS/ZnO/Ag group (0.88 mm) reflects the more consistent activity across ratios. The measured effect size was large (Cohen's d = 1.20) that indicates a significant difference in antibacterial performance between the two formulations. However, as the available dataset is based on the summarized inhibition zone values and not independent measurements on the replicate level, these statistical comparisons should be considered as exploratory and descriptive rather than confirmatory.

In a broad sense, the antibacterial response of the CS/ZnO/Ag nanocomposite was similar for the three bacterial species tested. The mean inhibition zones of *S. aureus*, *E. coli,* and *K. pneumoniae* were similar, suggesting that the nanocomposite possessed antibacterial activity against both Gram-positive and Gram-negative bacteria without any obvious species-specific bias. In general, the results confirm that CS nanoparticles present an antibacterial effect depending on their concentration and that the enrichment with ZnO and Ag nanoparticles improves the antibacterial effect, especially at the 3:1 ZnO:Ag ratio.

As demonstrated by Li et al. (2010), the CS/ZnO nanocomposite shown has antibacterial properties towards *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. ROS production is dependent on the nanoparticles' surface area; a larger surface area results in a greater ROS level (Shi et al., 2014). Due to its abundance of amine and hydroxyl groups, CS has a significant affinity for metal ions (Ardean et al., 2021). The combination of CS/Ag/ZnO shows stronger antibacterial activity than chitosan, according to Simonescu et al. (2014), suggesting that ZnO and Ag boost chitosan's antimicrobial properties. The most viable approaches are (i) adding chitosan to the NPs' surface, which causes "steric stabilization" and decreases agglomeration, increasing the NPs' effective concentration; and (ii) strengthening the bonds between the bacterial negative charges and the chitosan's positive charges (Yuan et al., 2024).

The epidemiological distribution of the bacterial isolates obtained in this study deserves careful consideration. The most common bacterium isolated from wound infections was *S. aureus* (16%), which is of clinical importance as reported earlier. The most frequent pathogen isolated from infected wounds was *S. aureus* (40%–60% of all isolates from various wound types) (Puca et al., 2021). The somewhat lower prevalence found in this study is probably due to the differences in infection ecology and patient demography in different areas and institutions. The second most common isolate was *K. pneumoniae* (11%), which is significant from the point of view of antibiotic resistance. It is an opportunistic Gram-negative bacterium, a member of the ESKAPE group of high priority pathogens and is highly associated with multi-drug-resistant nosocomial infections including carbapenem-resistant strains (Pareek et al., 2021). The inclusion of *K. pneumoniae* in the panel of test organisms is a great improvement for the clinical relevance of the antibacterial evaluation, in addition to studies that only deal with *E. coli* and *S. aureus*.

The pure CS NPs showed dose-dependent antibacterial activity. Quantitative determination of the bioactivity was dose-dependent with inhibition zones from 5 mm (0.25 g) to 10 mm (1.0 g) against the three pathogens tested. Mechanistically, this behaviour may be attributed to the protonation of chitosan amino groups ($-NH_3^+$) under acidic conditions, which strengthens electrostatic interactions with negatively charged teichoic acids and lipopolysaccharides on bacterial cell surfaces, ultimately disrupting membrane integrity (Ke et al., 2021). However, the inhibition values converge at the highest CS concentration (10 mm, all three organisms at 1.0 g), indicating that the extra outer membrane barrier of Gram-negative organisms can be overcome by sufficient chitosan concentration. This is a practically important finding because *K. pneumoniae*, a notoriously resistant Gram-negative pathogen, showed equal susceptibility to *S. aureus* at a high concentration.

The increase of the antibacterial activity of CS/ZnO/Ag ternary nanocomposites compared to the pure CS NPs can be explained in terms of several concurrent bactericidal mechanisms. ZnO and Ag NPs are known to generate reactive oxygen species (ROS) such as superoxide radicals (O2−) and hydroxyl radicals (•OH). This results in oxidative stress, that induces irreversible damage to bacterial DNA, lipid peroxidation and protein denaturation (Sklodowski et al., 2023). The chitosan matrix enables dispersion of nanoparticles in the ternary system that, results in maximum effective surface area and ROS yield (Shi et al., 2014). Also, Ag+ ions released from Ag NPs have a direct bactericidal effect by binding to sulfhydryl groups of the essential bacterial enzymes and disrupting electron transport chain (Crisan et al., 2021). On the other hand, ZnO NPs damage the bacterial membrane integrity by direct physical contact, which has been proven to be very effective for *K. pneumoniae* in preventing cellular adhesion (Salem et al., 2015). Together, these different but complementary mechanisms provide a molecular basis for the observed synergy as per recent reviews on chitosan-metallic hybrid wound dressings (Halarnekar et al., 2023).

The uniqueness of the present study is emphasized in that these results are corroborated in the wider nanomedicine literature. Similar values for inhibition zones against *S. aureus* and *E. coli* have been reported for sponge-like composites of similar nature, i.e., chitosan/Ag/ZnO composites (Alven & Aderibigbe, 2024). This is quite similar to the nanoparticle loadings in the present study (Bagheri et al., 2022). Pino et al. (2023)

performed a comprehensive review on the application of ZnO-containing biocomposites in wound healing and highlighted that the incorporation of ZnO into biopolymer matrices consistently enhances the antibacterial activity and wound closure rates, suggesting a possibility for the clinical translation of these hybrid materials. The enhanced activity of ZnO nanoparticles against *S. aureus* has been further confirmed by Hao et al. (2024) who found that ZnO NPs not only inhibited the bacterial growth but also suppressed the expression of biofilm genes (icaA, icaD, fnbA) in *S. aureus* isolates. This mechanism is particularly pertinent in the context of the biofilm forming propensity of clinical wound isolates. Unlike standardized reference strains, the present study provides direct validation against clinical wound isolates from hospitalized patients in Al-Najaf Al-Ashraf, Iraq. The demonstration of antibacterial activity is more meaningful in real world wound management settings as clinical isolates are more likely to contain acquired resistance determinants. Moreover, the systematic study of three different ZnO:Ag ratios is a methodological contribution that is not always considered in previous studies, which usually show only one composite formulation without optimizing the composition.

Several limitations of the current study design should be acknowledged. The agar well diffusion method, though operationally robust and extensively used, is a qualitative screening tool that does not offer minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) or bacteriostatic-versus-bactericidal characterization. Recent studies on nanocomposites with similar functions have shown that the antibacterial activity in vitro may differ from in vivo effects due to the influence of wound microenvironment such as pH change, exudate composition and different tissue contact mechanisms (Shu et al., 2023; Halarnekar et al., 2023). Assessment of antibiofilm activity remains an important gap. Abdelraheem et al. (2021) showed that sub-MIC concentrations of ZnO NPs markedly reduced *S. aureus* biofilm formation, achieving up to 76.47% inhibition, and downregulated biofilm-associated genes (icaA, icaD, and fnbA) in clinical isolates from burn wounds. These findings suggest that the CS/ZnO/Ag system examined here may also possess antibiofilm properties that were not evaluated in the present study and warrant targeted future investigation, particularly given the key role of biofilm-forming pathogens, including *K. pneumoniae*, in the progression of wound infections to chronicity. Future studies addressing these parameters would greatly strengthen the translational basis for clinical deployment of CS/ZnO/Ag nanocomposites.

In the wider context of the global antimicrobial resistance crisis, in which the WHO has identified drug-resistant bacteria as one of the most serious threats to public health, the multi-mechanistic action of CS/ZnO/Ag nanocomposites (membrane disruption, ROS generation, and metal ion release) represents a bactericidal strategy fundamentally less prone to resistance development than conventional antibiotics that target specific molecular pathways (Yang et al., 2021). Today, alternative strategies, including those based on nanoparticles, against ESKAPE pathogens are increasingly recognized as essential tools in the clinical armamentarium against MDR infections (Mulani et al., 2019). It makes CS/ZnO/Ag nanocomposites not just an additional agent but a potential first-line biomaterial in the management of wound infections, especially in hospitals where MDR organisms are common.

4. Conclusion

The present study shows that the chitosan-based nanocomposites with zinc oxide and silver nanoparticles (CS/ZnO/Ag) show significant enhancement of broad-spectrum antibacterial activity as compared to pure chitosan nanoparticles against clinically isolated wound isolates of *S. aureus*, *E. coli*, and *K. pneumoniae*. The best formulation was 3:1 (ZnO:Ag) with 0.15 wt% nanoparticle loading, where the highest inhibition zone (11 mm against *K. pneumoniae*) was achieved, which was a significant improvement over the performance of pure CS NPs (maximum 10 mm). This increased efficacy is mechanistically explained by synergistic multi-pathway bactericidal action: ROS mediated oxidative stress, direct disruption of essential bacterial enzymes by Ag+ ions, membrane permeabilization by ZnO and chitosan mediated enhancement of nanoparticle dispersion and electrostatic interaction with bacterial membrane. In terms of the contributions to existing literature, the current study makes several novel advances. First, it provides empirical antibacterial data against clinical wound isolates from Al-Najaf Al-Ashraf hospitals in Iraq, a geographic and clinical setting under-represented in the published nanomedicine literature, thereby adding regional epidemiological specificity and translational relevance beyond usual reference-strain-based investigations. The second aspect is the systematic comparison of three ZnO:Ag molar ratios, offering compositional optimization data that is not generally reported in previous studies of analogous systems (Simonescu et al., 2014; Yuan et al., 2024), thus establishing the 3:1 ratio as the benchmark formulation for further development. Third, the choice of *K. pneumoniae* as the principal test organism lends clinical weight to the findings as this is an ESKAPE-group nosocomial pathogen with documented carbapenem resistance. Future studies should mainly be directed to MIC/MBC determination, in vitro cytotoxicity profiling against human keratinocytes and fibroblasts, antibiofilm activity assessment, and finally in vivo wound healing efficacy evaluation in suitable animal models. With the integration of these data streams, CS/ZnO/Ag nanocomposites would be evidence-based candidates for clinical wound dressing development amid the escalating global antimicrobial resistance crisis.

Ethics committee approval: Ethical approval for this study was obtained from the Central Committee for Bioethics at the University of Kufa (Iraq). The committee reviewed the application at its meeting on September 28, 2022 and approved the project titled "Antimicrobial of Chitosan / ZnO / Ag Nanocomposite for Wound Healing". The research was conducted in accordance with the approval of the relevant ethics committee.

Conflict of interest: The authors declare that there is no conflict of interest.

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