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Production of Nanocomposite Silver Packaging using Solution Blending Method for the Supplement of Antibacterial Coating

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Abstract

Background and Objective: The objective of this study was to assess antimicrobial effects of silver nanoparticles on Gram-positive and Gram-negative bacteria that used in preparing silver nanocomposite with the antibacterial characteristics using solution method. Moreover, the aim of the current study was to produce antimicrobial silver nanocomposites for food coating with their effects on a wide range of bacteria.

Material and Methods: To assess antibacterial characteristics of silver nanoparticles, several steps were carried out. First, nanoparticles were synthesized through a chemical reduction method using NaBH4 and then analyzed using x radiation diffraction, ultraviolet and visible spectroscopic analysis, dynamic light scattering and scanning electron microscopy nanometric assays. Then, *Staphylococcus aureus* and *Escherichia coli* were used as Gram-positive and Gram-negative bacterial indicators. Minimum inhibitory concentration, minimum bactericidal concentration and inhibition zone levels were measured. Nanocomposite was produced using solution blending method and its antibacterial characteristics were assessed using inhibition zone method.

Results and Conclusion: Results indicated that silver nanoparticles with 20 and 50 $\mu g.l^{-1}$ concentrations included inhibitory effects on *Staphylococcus aureus* and *Escherichia coli*, respectively. Furthermore, concentrations of 40 to 60 $mg.l^{-1}$ included lethal effects on *Staphylococcus aureus* and *Escherichia coli*, respectively. Based on the results, the highest antibacterial effects were observed on Gram-positive *Staphylococcus aureus*. In inhibition zone assays, a 3-5 mm zone was seen around the silver nanoparticle discs in cultures of the microorganisms. In the inhibition zone assay of the produced nanocomposites, the zone was expected regarding the concentrations. Results were calculated in three repetitions and the value estimated through ANOVA was significant when $p<0.0001$. It has been concluded that silver nanoparticles are useful in Gram-positive and Gram-negative bacteria for the inhibition and destruction. Moreover, it has been verified that using the method includes great effects on antibacterial characteristics of the nanocomposites.

Conflict of interest: The authors declare no conflict of interest.

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S. aureus

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1. Introduction

In recent years, use of metal nanoparticleshas increased due to the resistance of pathogenic microorganisms (bacteria, fungus and viruses) against conventional antimicrobials. General concerns on the safety and quality of foods, particularly marine foods, during storage and stocking have led the microbial growth control a fundamental part of the distribution and storage chain of such products [1-4]. Based on various studies and assessment of silver nanoparticle function mechanism against microorganisms, their use as antimicrobial agents, especially in the food and medical industries, can be one of the novel solutions for conquering problems caused by the pathogenic microorganisms. Food products are infected by various microbial agents during production processesss. Infections may occur in formulation of ingredients, using chemical additives and posing high pressure and flash

pasteurization. Harmful materials likely enter food formulation during this process, or chemical reactions may occur with dangerous outcomes to humans.

If food products are in contact with contaminated surfaces or surfaces with metal ions during the production processes, this can endanger consumers' health. Therefore, use of proper antimicrobial packaging based on bio-polymers is nowadays highly interested due to being biodegradable, lack of collection of various synthesized materials in the natural ecosystem and improvement of mechanical and viscoelastic characteristics as well as appropriate antimicrobial characteristics [5]. Liao et al. studied the antibacterial activity and action mechanisms of silver nanoparticles (AgNPs) against *Pseudomonas aeruginosa* resistant to several medicines. In that study, use of morphological changes and assessment of active oxygen and activity of enzymes in the bacteria when exposed to AgNPs as well as reporting minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) showed the potential antibacterial effects of AgNPs on the bacteria [6]. Jo et al. investigated antibacterial characteristics of polyethylene and polypropylene nanocomposite films using AgNPs. First, nanocomposites were prepared using melting method and extruder. Based on the results, these nanocomposites included a 99.9% destructive effect on *Staphylococcus aureus* and *Escherichia coli* bacteria. This result showed well that using these nanocomposites could be effective and efficient in food packaging [7]. Furthermore, researchers synthesized degradable films with mixed clay and polyvinyl alcohol (PVA). They used these films against essential food pathogens such as *Salmonella typhimurium* and *Staphylococcus aureus*. Their results could reflect high antimicrobial effects of these nanoparticles, their mechanical characteristics and appropriate flexibility caused by PVA, as well as biodegradability of these films, which were verified through burring assaessment of them in the ground. To show efficiency of the highlighted packaging bags, shelf life of the chicken sausage samples was compared with that of regular polyethylene bags. Results showed enhancements in shelf life by decreasing microbial loads [7].

Mathew et al. synthesized nanocomposites, combining clay and biodegradable PVA. Based on their findings, the combined nanocomposite films included sufficient antimicrobial characteristics against food pathogens such as *S. typhimurium* and *S. aureus* and higher mechanical characteristics such as resistance against water and light transmission, compared to control films. The soil burial assay revealed that the nanocomposites degraded within 110 d and hence were considered biodegradable. Then, nanocomposite combined films were included in the bags used for keeping chicken sausages, which resulted in decreases in microbial loads compared to the control polyethylene bags and were much more effective in increasing shelf life of the chickens [8]. Findings from their study were similar with those of the the current study.

Liao et al. studied antibacterial characteristics and mechanisms of AgNPs against *P. aeruginosa* resistant to drugs. In this study, antimicrobial effects of AgNPs on resistant clinical isolates against *P. aeruginosa* with MIC and MBC were investigated. Morphological changes were observed in *P. aeruginosa* resistant against drugs under transmission electron microscopy (TEM). Distinct protein highlighted in the proteomics approach was studied quantitatively and production of reactive oxygen species was assessed using 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) coloring. Activity of superoxide dismutase (SOD), catalase and peroxidase was chemically assessed and apoptosis effects were studied through flow cytometry. Findings revealed that AgNPs included strong inhibitory effects on *P. aeruginosa* resistant against the antimicrobials with MIC of 1.406-5.625 $mg.ml^{-1}$ and MBC of 2.81-5.62 $mg.ml^{-1}$. Results of TEM revealed that AgNPs could penetrate resistant bacteria and disrupt their structure. Furthermore, quasi-apoptosis in bacteria affected by AgNPs was significantly higher. General findings and the estimated *p*-value ($p<0.01$) revealed strong antibacterial effects of AgNPs on multiresistant *P. aeruginosa* [9]. Active packaging incorporating AgNPs becomes popular due to its efficacy in combating foodborne pathogens. This technology uses AgNPs directly embedded in the packaging materials or adsorbed as ions, offering a safe effective antimicrobial shield. Recent approvals by the European Food Safety Authority (EFSA) for specific silver compounds further facilitates broader implementations [10].

This study investigated antibacterial effects of AgNPs on Gram-positive and Gram-negative bacteria and produced silver nanocomposites with appropriate antibacterial characteristics using solution blending. As previously stated, the present experimental study investigated use of AgNPs synthesized via chemical resuscitation method by assessing their MIC, MBC and inhibition zone against *S. aureus*, *E.coli* and *Candida albicans*, leading to decreases of food spoilage and enhancement of food shelf life. Moreover, their antimicrobial effects were studied as alternatives to antimicrobials. The aim of this study was to investigate antibacterial effects of AgNPs on Gram-negative and Gram-positive bacteria and produce silver nanocomposites with appropriate antibacterial characteristics using solution blending method.

2. Materials and Methods

2.1. Synthesis and characterization of silver nanoparticles

The AgNPs were synthesized using chemical reduction method with sodium borohydride ($NaBH_4$). Dynamic light scattering (DLS) verified the particle sizes within the

desired range of 25-40 nm. The X-ray diffraction (XRD) analysis revealed crystal structure of the materials, while UV-VIS spectroscopy provided information on nanoparticle size and homogeneity. Additionally, scanning electron microscopy (SEM) visualized morphology of the synthesized nanoparticles.

2.2. Antimicrobial activity assessment

Culture media and autoclaves were sterilized. Each experimental tube included 5 ml of culture media, 100 mg of bacteria/fungi and calculated concentrations of AgNPs. Triplicate experiments were carried out.

2.3. Minimum inhibitory concentration and minimum bactericidal concentration assessments

Microdilution assay in gamma tubes was used. Nutrient broth was used for *S. aureus* and *E. coli* and Sabouraud dextrose (SD) broth for *C. albicans*. Standardized inocula (100 µl) were added to the broths and incubated at 37 °C for 24 h. The MIC was assessed as the lowest concentration inhibiting visible growth. The MBC included plating 100-µl aliquots onto agar media (nutrient agar for bacteria and SD agar for fungi) and incubating at 37 °C for 24 h. Moreover, MBC was defined as the lowest concentration demonstrating no microbial growth or less than three colonies (99-99.5% killing).

2.4. Nanoparticle synthesis method

First, sodium borohydride was dissolved in water (ice bath) and mixed with polyvinylpyrrolidone (PVP). Silver nitrate solution was then added to the mixture, which changed the color from yellow to orange, brown and then black. This was then stirred quickly (~1500 rpm) at 50–60º C, which created clods. Drops of silver nitrate (0.001 M) were added to sodium borohydride (0.002 M) set in the ice bath, which changed color of the final product to yellow. This color became darker over time. To increase stability of the product, 1% PVP was added to the solution, which changed color of the product to pale orange-red. Concentration of the colloidal nanosilver was 6 $mg.ml^{-1}$ and based on the UV-VIS assay, size of the particle was 25–40 nm. The quantity of PVP used for increasing stability was 3 $mg.ml^{-1}$.

2.5. Nanocomposite production through solution blending

In brief, 500 ml of the polymer PVA were divided into five beakers with volume of 0.28, 0.42, 0.83, 1.67 and 2.5 ml. Then, AgNPs were added to 12.5 25, 50, 100 and 150 ml with a concentration of 6 $mg.ml^{-1}$. These were stirred on a stirrer heater at 50 ºC for 24 h until volume of the solution reached 20 ml. The final product was poured into a Petri dish and set in the oven for 24 h to dry. Then, the final composite with similar thickness and appropriate level of flexibility was ready.

2.6. Analysis of the size of nanoparticles using dynamic light scattering method

In general, DLS is a technique used to assess particle sizes in solutions and suspensions. In this method, specialized devices analyze the motion of particles while they are suspended in a liquid. It provides a rapid and non-destructive way to assess particle sizes, ranging from nano to micrometers. For example, researchers transferred nanosilver colloids into the DLS device cell. The subsequent analysis estimated the particle size. Specifically, 5 ml of nanosilver colloid were analyzed at 25 °C with a laser strength of 60%.

2.7. UV-VIS analysis

Characteristics of photons on samples and measuring the rate of passage or absorption (rate of adsorption or reflectance of light) in various wavelengths ranging 200-1100 nm. Results of the assay were presented in a typical surface absorption plasmon at 420 nm achieved from the AgNPs.

2.8. Scanning electron microscopy analysis

The SEM is an exceptionally well-suited method for the study of nanoparticle structure and it depicts the size of AgNPs in ranges from 10 to 100 nm. No agglomeration was seen in nanoparticles, showing stabilization of the nanoparticles.

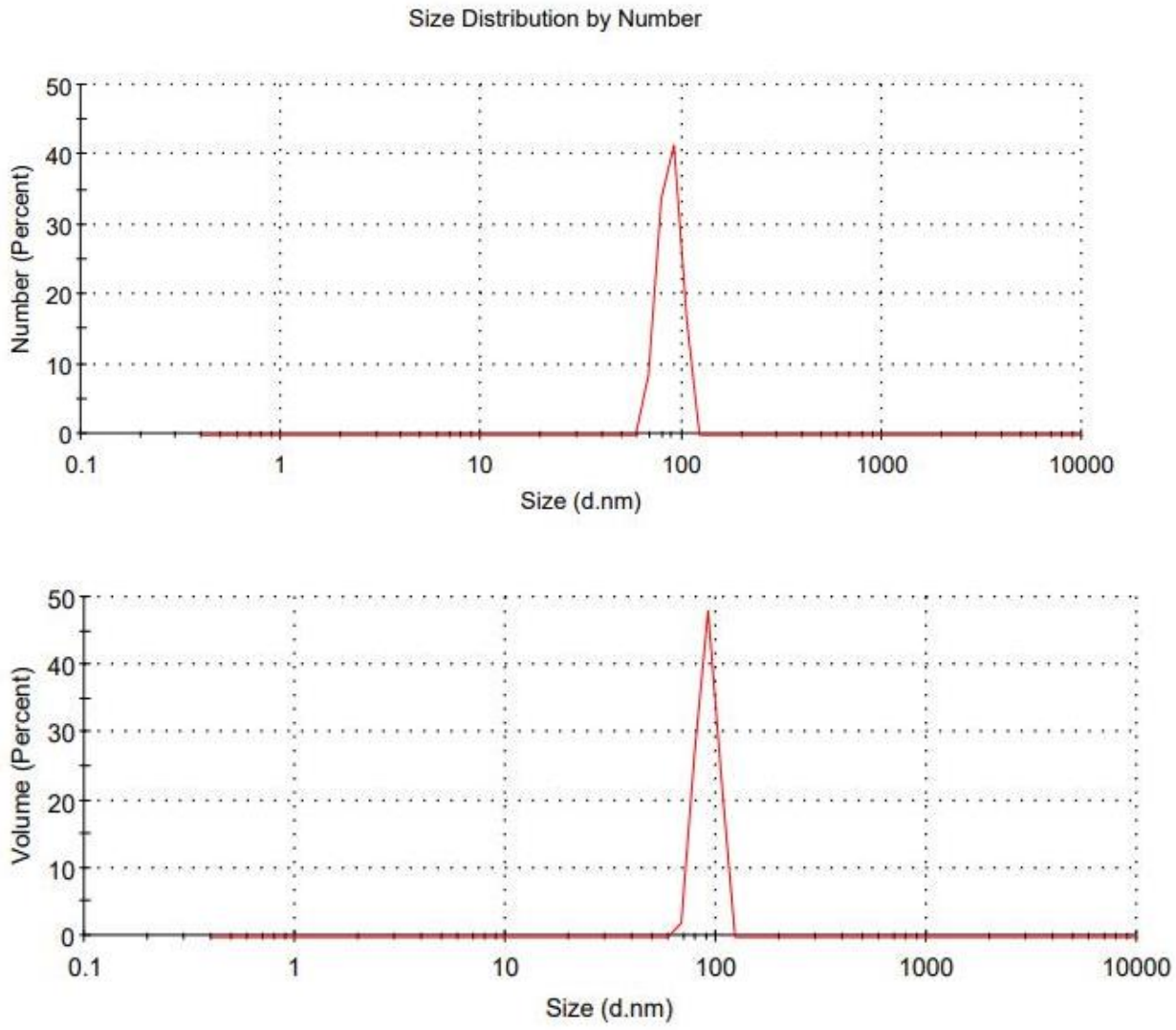
3. Results and Discussion

3.1. Analysis of the nanoparticle characteristics

The DLS results from Fig. 1 (a, b, c) revealed essential information on AgNPs. Based on the figure, AgNPs demonstrated the following characteristics. Number distribution, approximately 41% of the particles within specific size ranges; volume distribution, nearly 48% of the particles contributing to the overall volume; intensity distribution, significantly 92% of the scattered light intensity originating from the specific particle sizes.

Additionally, Fig. 2 shows a SEM image of AgNPs synthesized through chemical reduction. These nanoparticles exhibited spherical shapes and included a size range of approximately 25 to 40 nm.

Furthermore, Fig. 3 presents the UV-VIS diagram, providing further characterizations of the AgNPs. To assess structural characteristics of the nanocomposite films, XRD analysis was used. Nanosilver samples were irradiated with Cu-Kα radiation (λ = 54.1 Å) using X-ray spectrometer operating at 40 kV and 30 mA. The resulting XRD pattern provided valuable information on the crystalline phases and crystallographic orientation within the nanocomposite films (Fig. 4).



B. Nanoparticles with a size of 145.8 nm

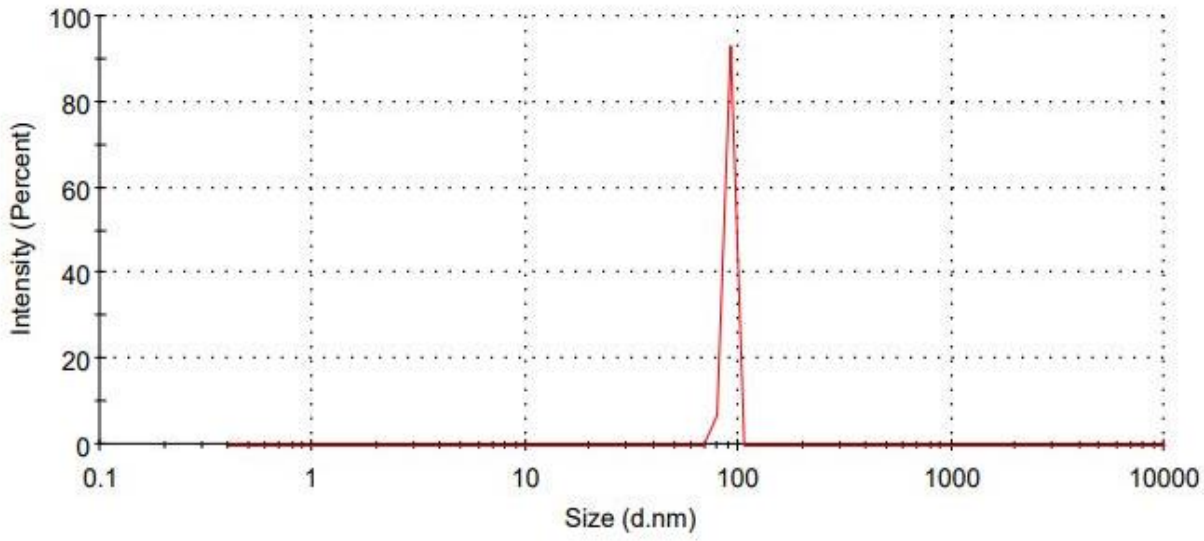


Figure 1. Dynamic light scattering diagram of the produced silver nanoparticles with various sizes: **a,** 121.9, **b,** 145.8 and **c,** 150.3

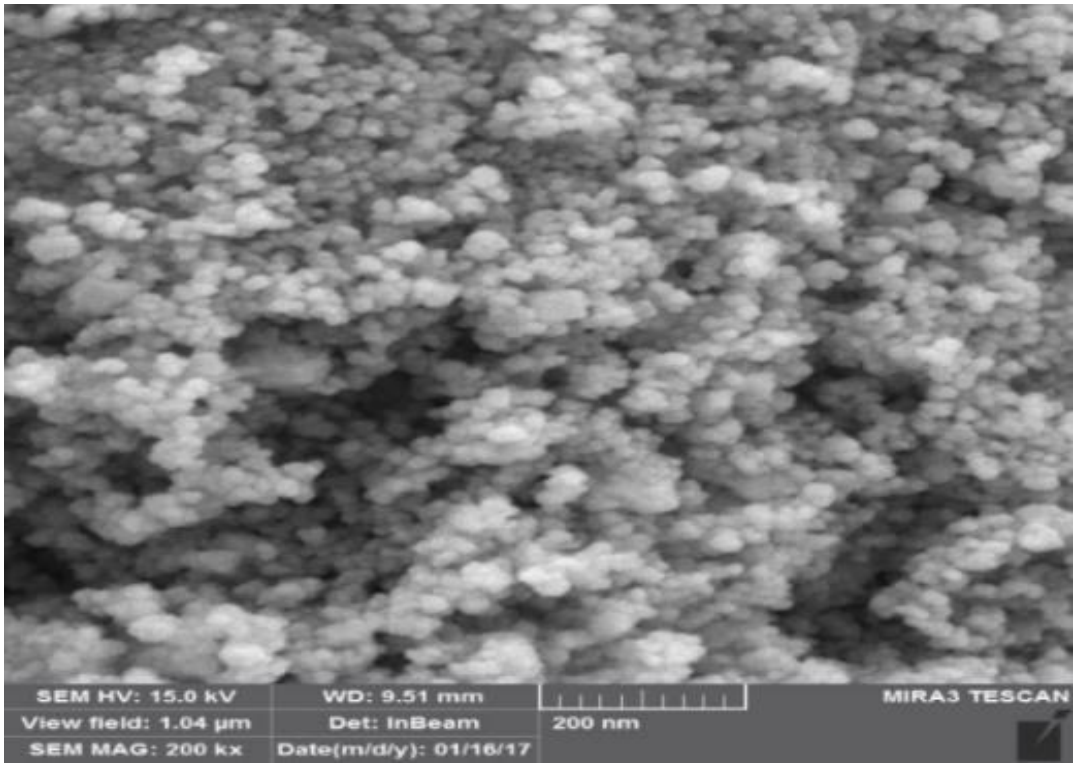


Figure 2. Scanning electron microscopy of the silver nanoparticle synthesis using $NaBH_4$

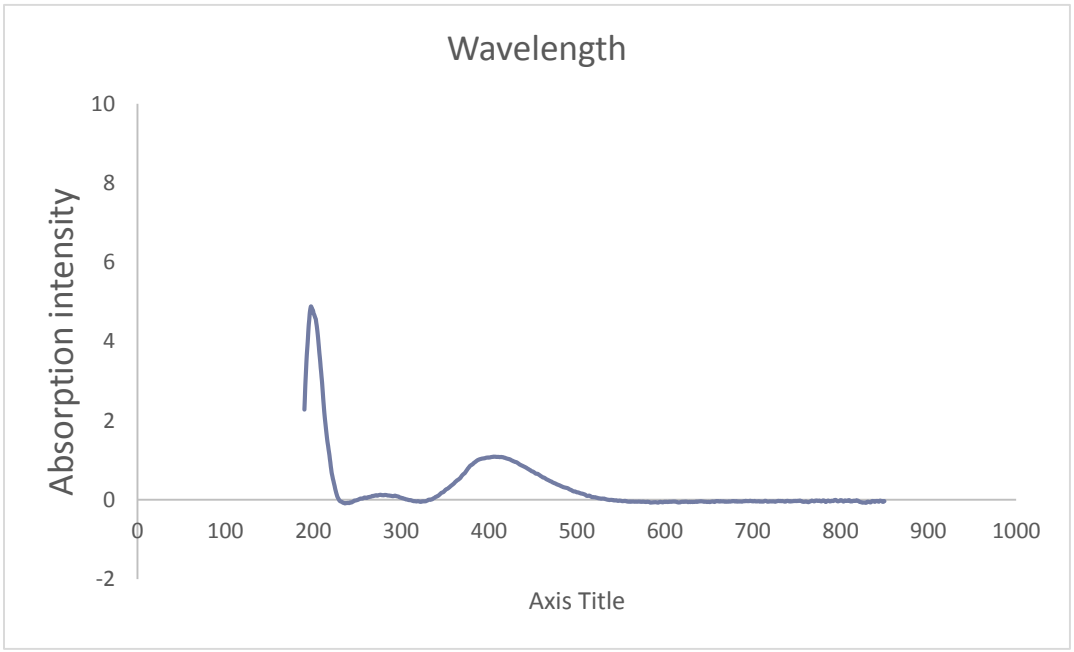


Figure 3. The UV-VIS diagram of the produced silver nanoparticles

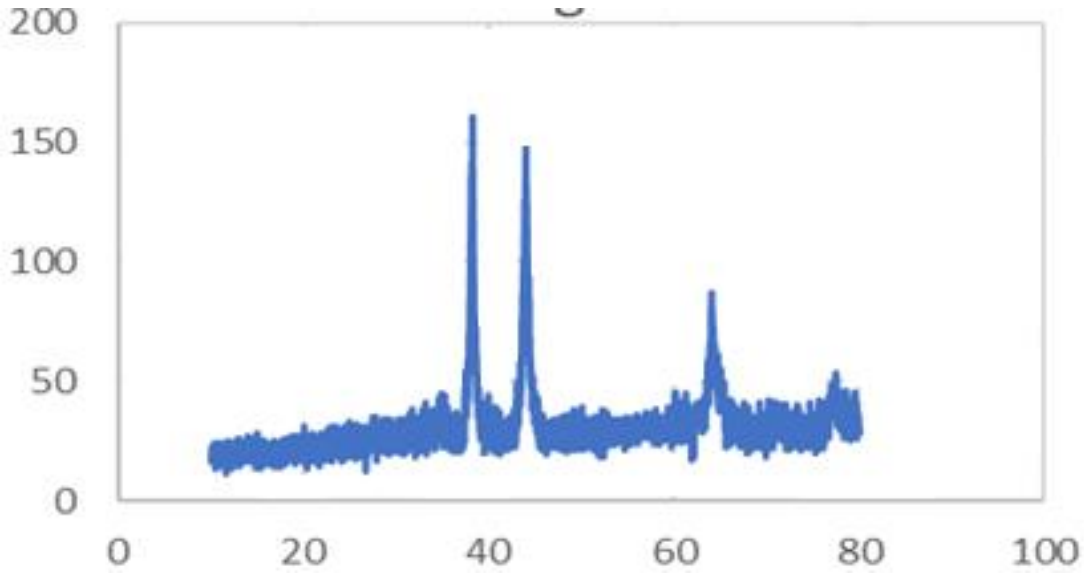


Figure 4. The X-ray diffraction diagram of the produced silver nanoparticles

3.2. Minimum inhibitory concentration and minimum bactericidal concentration assessments

The MIC and MBC of the biosynthesized nanoparticles were assessed against various pathogens. Nanoparticles showed potential antibacterial activities against *E. coli* (MIC, 50 µg.ml^{-1} and MBC, 70 µg.ml^{-1}) and *S. aureus* (MIC, 25 µg.ml^{-1} and MBC, 45 µg.ml^{-1}). However, nanoparticles demonstrated weaker antifungal activities against *C. albicans* (MIC, 350 µg.ml^{-1} and MBC, 380 µg.ml^{-1}). Results suggested potentials of these nanoparticles as broad-spectrum antimicrobials. Although further optimizations may be necessary for the enhanced antifungal efficacies (Fig. 5).

3.3. Antimicrobial susceptibility assay for the assessment of inhibition zone diameters (disk diffusion)

To assess antibacterial and antifungal activities of the AgNPs, inhibition zone assay was used via diffusion disks impregnated with various concentrations (200 and 6000 µg.ml^{-1}). Three microorganisms were assessed, including *S. aureus, E. coli and C. albicans*. Isolated bacterial colonies of *S. aureus* and *E. coli* were suspended in sterile serum, creating a homogenous solution. This solution was then streaked onto agar plates using sterile swabs. Blank disks loaded with either AgNPs or control antibiotics (amikacin for bacteria and itraconazole for fungi) were transferred onto the inoculated plates. Following incubation at 37 °C for 24 h, diameters of the resulting inhibition zones around the disks were measured using caliper. For the nanocomposite disks, another experiment was carried out, where blank disks were punched out and loaded with various nanoparticle concentrations. These disks were then added to the bacterial cultures and the inhibition zones were measured as described. Results of this study provided information on the potentials of the AgNPs as antimicrobial agents against various pathogens (Table 2).

Findings of MBC and MIC assays verified that by prohibiting microorganisms, AgNPs could increase the shelf life of foods (Table 1). Results revealed the higher effects of AgNPs on Gram-positive *S. aureus*, compared to Gram-negative *E. coli*. According to Abbaszadegan et al., the major reason for differences in antibacterial effects of Gram-positive and Gram-negative bacteria included the quantity of peptidoglycan in the bacteria cell wall. Ggram-positive strains included further peptidoglycans in their cell walls, compared to those Gram-negative strains did, allowing AgNPs to include extended inhibition zones for these strains.

This study demonstrated effectiveness of AgNPs in extending food shelf life by inhibiting microbial growth. Studies, including those by Abbaszadegan et al. and Eslami et al., highlighted the nanoparticle efficacy against various bacteria, with Gram-positive strains such as *S. aureus* exhibiting a greater susceptibility, compared to that Gram-negative *E. coli* doing. This difference was attributed to the thicker peptidoglycan layer in Gram-positive bacterial cell walls, offering a larger target for AgNPs.

Researchers investigated the potential of AgNPs to combat microbes in various settings, including food preservation. In a study by Eslami et al. (2016), effectiveness of AgNPs in preserving saffron was investigated. Various concentrations of nanoparticles were incorporated into packaging materials and the microbial loads on the saffron were monitored over time.

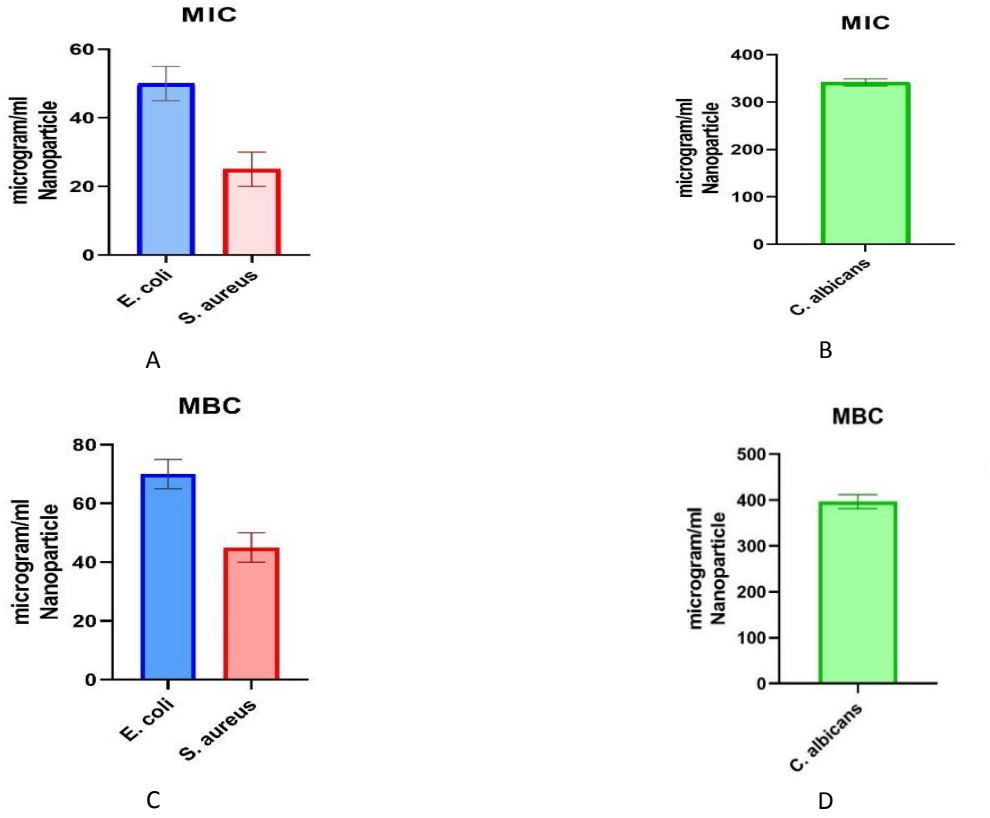


Figure 5. Statistical findings of the minimum inhibitory concentration assay using ANOVA

Table 1. Estimated nanosilver concentrations for MBC, MIC and MFC

Bacteria/fungi	MBC/MFC result µg.ml^{-1}	MIC µg.ml^{-1}
Escherichia coli	70	50
Staphylococcus aureus	45	25
Candida albicans	380	350

† MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, MFC: Minimum Fungicide Concentration

Table 2. Results of the inhibition zone assay for silver nanoparticles and nanocomposites

The results of inhibition zone tests in silver nanoparticle					
Bacteria/fungi	12 µg.ml^{-1} Silver nanoparticles	24 µg.ml^{-1} Silver nanoparticles	5 µg.ml^{-1} Penicillin	5 µg.ml^{-1} Amikacin	5 µg.ml^{-1} Itraconazole
Escherichia coli	2.5 mm	2.5 mm	4 mm	8 mm	-
Staphylococcus aureus	2 mm	3 mm	4 mm	7 mm	-
Candida albicans	-	0.5 mm	-	-	0.5 mm
The results of inhibition zone tests in the nanocomposite					
Bacteria/fungi		12 µg.ml^{-1} Silver nanoparticles		24 µg.ml^{-1} Silver nanoparticles	
Escherichia coli		3mm		4 mm	
Staphylococcus aureus		2.5-4 mm		3-5 mm	
Candida albicans		1 mm		1.5 mm	

Results showed significant decreases in microbial growth, particularly at higher nanoparticle concentrations, highlighting the potential of this technology for extending the shelf life of food products [10]. Antibacterial activity of the AgNPs against hospital-acquired antibiotic-resistant strains of *P. aeruginosa* was assessed by Salomoni et al. [11]. Commercial nanoparticles effectively inhibited bacterial growth at specific concentrations, suggesting their potentials as tools to combat challenging infections. Further studies such as that by Alsharqi et al. deeper investigated mechanisms; by which, AgNPs exert their antimicrobial effects. Their *in-vitro* experiments demonstrated that nanoparticles interacted with bacterial cell membranes, ultimately suppressing bacterial growth. Significantly, this effect was observed against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria, although various degrees

of susceptibility were observed [12]. Similar to the current findings, these studies collectively present encouraging evidence for the use of AgNPs as a novel antimicrobial strategy. Further studies are needed to assess their potential uses and safety profiles.

The SEM images of the treated bacteria cells revealed significant morphologic changes in the cell membranes after processing with AgNPs. Results indicated strong antibacterial reactivities in AgNPs that could inactivate harmful and pathogenic microorganisms [12]; similar to results of the present study. Previous findings showed that AgNPs directly attacked the cell membrane of bacteria, causing significant morphological changes [12]. This verified strong antibacterial activities of these nanoparticles, further supporting their potentials to combat harmful microorganisms.

Yan et al. [13] investigated broadly mechanisms of action using proteomics approaches, revealing 59 proteins affected by silver interactions. Interestingly, silver interacted with several membrane proteins and triggered production of ROS within the bacteria. This ROS production ultimately damaged the cell membrane, leading to bacterial death. These findings were perfectly similar to findings from the current study and other studies, highlighting the potential antimicrobial roles of AgNPs. Additionally, Pooyamanesh et al. [14] successfully incorporated AgNPs into food packaging materials, demonstrating their effectiveness against various foodborne bacteria such as *E. coli* and *S. aureus*. This evidence strongly suggests that AgNPs include tremendous potentials in combating harmful bacteria, opening doors for novel uses in food preservation. However, further studies are necessary to fully understand their safety and optimize their effectiveness for various uses.

4. Conclusion

This study has validated potentials of AgNPs and their nanocomposites for antimicrobial uses in food packaging. Findings from MIC, MBC and inhibition zone assays consistently have demonstrated their effectiveness against various bacteria, especially Gram-positive strains. These provide direct benefits for food preservation, extending shelf life while eliminating needs of harmful chemical additives. Integrating AgNPs into food packaging offers more than a chemical-free alternative; it presents a multifaceted solution with far-reaching benefits. First, it develops organic food production by effectively combating bacteria without conventional preservatives, fostering trust and enhancing food quality. Second, their significant antibacterial ability originates from their high surface areas and positive charges, disrupting the bacterial membranes and significantly extending food shelf lives. Third, the chemical resuscitation method allows for precise control of nanoparticle sizes, tailoring their interaction with specific bacteria for optimized performance. Fourth, the suggested solution blending method improves cost-effectiveness, making this innovative technology readily accessible. While further studies are critical to understand long-term effects and ensure responsible implementation, these diverse advantages offer AgNPs as a promising solution for the challenges of food preservation. While Gram-positive bacteria have demonstrated greater susceptibilities due to their cell wall structures, effectiveness of AgNPs even at authorized low concentrations and their minimal risks of release into foods further highlight their potentials as safe sustainable alternatives to the available antimicrobial agents. This study pioneers further investigations and optimization of silver nanoparticle-based food packaging solutions, offering a promising path towards enhanced food safety and decreased environmental adverse effects. However, it is important to acknowledge needs of continuous studies to comprehensively understand potential long-term effects of the current technology.

5. Acknowledgements

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6. Conflict of Interest

The authors declare no conflict of interest.

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تولید بسته بندی نانوکامپوزیت نقره به روش مخلوط کردن محلول برای تکمیل پوشش ضدباکتریایی

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چکیده

سابقه و هدف: هدف از این تحقیق بررسی اثر ضد میکروبی نانوذرات نقره بر روی باکتری‌های گرم مثبت و منفی تولید شده برای تهیه نانوکامپوزیت نقره با خاصیت ضد باکتریایی زیست‌تخریب‌پذیر به روش محلول است. مطالعه حاضر با هدف تولید نانوکامپوزیت های نقره زیست تخریب پذیر ضد میکروبی برای تولید پوشش غذایی و تاثیر بر طیف وسیعی از باکتری ها می باشد.

مواد و روش ها: برای ارزیابی ویژگی‌های ضد باکتریایی نانوذرات نقره، تحقیق در چند مرحله انجام شد. ابتدا نانوذرات از طریق روش احیای شیمیایی با استفاده از NaBH4 تولید شد و سپس با استفاده از سنجش‌های نانومتری پراکنش پرتو ایکس[۱]، طیف بینی مادون قرمز و مرئی[۲]، پراکنش پویای نور[۳] و میکروسکوپ الکترونی روبشی[۴] مورد تجزیه و تحلیل قرار گرفت. سپس از *استافیلوکوکوس اورئوس* و *اشرشیا کلی* به‌عنوان باکتری‌های شاخص گرم مثبت و منفی استفاده شد. حداقل غلظت بازدارندگی[۵]، حداقل غلظت کشندگی باکتریایی[۶] و سطح منطقه مهار رشد (هاله مهار رشد) اندازه گیری شد. نانوکامپوزیت با استفاده از روش اختلاط محلول تولید و ویژگی‌های ضدباکتریایی آن با روش محدوده بازدارنده بررسی شد.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد که نانوذرات نقره در غلظت‌های ۲۰ $\mu g.l^{-1}$ و ۵۰ به‌ترتیب دارای اثرات بازدارنده بر *استافیلوکوکوس اورئوس* و *اشرشیا کلی* بودند. علاوه بر این، غلظت‌های ۴۰ $\mu g.l^{-1}$ تا ۶۰ به‌ترتیب دارای اثرات کشندگی بر *استافیلوکوکوس اورئوس* و *اشرشیا کلی* بودند. بر اساس نتایج، بیشترین اثرات ضد باکتریایی بر روی *استافیلوکوکوس اورئوس* گرم مثبت مشاهده شد. در آزمون محدوده بازداری، یک ناحیه ۳-۵ میلی‌متری در اطراف دیسک‌های نانوذرات نقره در کشت‌های میکروارگانیسم‌ها مشاهده شد. در آزمون محدوده بازداری نانوکامپوزیت‌های تولید شده، با توجه به غلظت‌های مورد استفاده نتایج قابل انتظار بود. نتایج سه تکرار محاسبه شد و آنالیز واریانس هنگامی‌که $P< ۰/۰۰۰۱$ بود نشان داد مقدار برآورد شده معنی‌دار است. نتیجه گیری شده است که نانوذرات نقره برای مهار و تخریب باکتری‌های گرم مثبت و منفی مفید هستند. علاوه بر این، تأیید شده است که استفاده از این روش اثرات زیادی بر ویژگی‌های ضد باکتریایی نانوکامپوزیت‌ها دارد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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[۱] X radiation diffraction (XRD)
[۲] Ultraviolet and visible spectroscopic (UV-VIS spectroscopic)
[۳] Dynamic light scattering (DLS)
[۴] Scanning electron microscopy (SEM)
[۵] Minimum inhibitory concentration (MIC)
[۶] Minimum bactericidal concentration (MBC)