



## Effects of Green Synthesized Silver, Copper and Silver-Copper Bimetallic Nanoparticles on Foodborne Pathogens

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### ABSTRACT

The present study aimed to investigate the antibacterial activity of silver (Ag), copper (Cu), and Ag–Cu bimetallic nanoparticles (BMNPs) synthesized at different concentrations via pomegranate peel extract usage as a reducing agent. Antibacterial activity was evaluated against *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus* by disk and well diffusion assays. AgNPs exhibited strong antibacterial activity across all tested pathogens, with the most pronounced effect observed at 0.05 M, particularly against *S. Aureus* ( $p < 0.05$ ). In contrast, CuNPs demonstrated lower antibacterial efficacy, with the strongest inhibition at 0.1 M, while significantly reduced activity was recorded at lower concentrations ( $p < 0.05$ ). Based on these findings, 0.05 M AgNPs and 0.1 M CuNPs were selected for the synthesis of Ag–Cu BMNPs at different ratios (1:1, 1:2, and 2:1, v/v). BMNPs exhibited enhanced antibacterial activity compared to their monometallic counterparts, indicating a synergistic effect between silver and copper ( $p < 0.05$ ). The antibacterial efficacy was found to be strain-dependent, with *S. aureus* showing greater sensitivity (1:1, v/v) to compositional variations, while *S. Typhimurium* and *L. monocytogenes* exhibited relatively stable inhibition across different ratios (1:2, 2:1, v/v). Overall, the results highlighted the superior antimicrobial potential of Ag–Cu BMNPs and suggest their promising application as novel antimicrobial substances to address the challenge of multidrug-resistant pathogens.

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## Introduction

Nanotechnology refers to a multidisciplinary area of research focusing on the control and utilization of nanoscale entities (1–100 nm), exploiting their unique morphological, chemical, and biological attributes for wide range of applications (Ajaz et al., 2024; Khan et al., 2022). In recent years eco-friendly or green synthesis methods have emerged as sustainable alternatives for nanoparticle production. Unlike conventional chemical or physical techniques that often employ hazardous reducing and stabilizing agents, green synthesis exploits biological materials such as plant extracts, microorganisms, and biopolymers as natural sources of reducing and capping agents (Lithi et al., 2025). Biologically derived compounds (polyphenols, flavonoids, and alkaloids) support stable nanoparticle formation under mild conditions while minimizing environmental and health risks (Eker et al., 2025; Zehra et al., 2025).

The exceptionally large surface-to-volume ratio of metallic nanoparticles (MNPs), which maximizes the number of reactive surface atoms, has led to their growing utilization in various sectors, including environmental

remediation, biomedical applications, packaging, electrocatalysis, and energy systems (Araya-Hermosilla et al., 2023; Jafarzadeh & Jafari, 2021; Wei et al., 2021). Although MNPs have demonstrated remarkable efficiency in a variety of applications, they are often limited by issues such as reduced stability, potential aggregation, and restricted multifunctionality (Burlec et al., 2023). Metallic nanoparticles are generally classified according to their composition into monometallic, bimetallic, and multimetallic systems (Huynh et al., 2020). Monometallic nanoparticles are composed of a single metal, while bimetallic and multimetallic nanoparticles incorporate two or more metals, respectively, enabling tunable physicochemical properties and broader functionality (Burlec et al., 2023). Bimetallic nanoparticles (BMNPs) in comparison to monometallic nanoparticles (MNNPs), often exhibit greater technological significance. Their properties and potential applications are determined not only by size and morphology but also by their specific metal composition and internal structure (Scala et al., 2022). The antimicrobial potential of MNPs against

bacterial, fungal, and viral pathogens has been widely documented, with proposed pathways including the induction of reactive oxygen species (ROS), membrane destabilization, intracellular impairment of proteins and DNA, and the release of metal ions that compromise key metabolic pathways (Lemire et al., 2013; Rai et al., 2009). The strong antimicrobial efficacy of silver nanoparticles (AgNPs) has been demonstrated against several microbial species. Because of their small size and large surface-to-area ratio, they readily associate with microbial membranes, inducing structural alterations that culminate in cell death. This makes AgNPs promising agents for applications in medicine, food preservation, and sanitation (Pal et al., 2007). Copper nanoparticles (CuNPs) demonstrate potent antimicrobial properties largely due to their capability of releasing copper ions, which interact with and disrupt microbial cell membranes. Membrane disruption impairs the cell barrier function of the cell, facilitating the outflow of essential intracellular molecules and triggering cell death. Moreover, CuNPs promote the generation of ROS, which subsequently cause oxidative stress and damage to essential biomolecules within microbial cells (Ren et al., 2009). Bimetallic silver-copper nanoparticles (Ag-Cu BMNPs) are of considerable interest since the combination of both metals often results in synergistic interactions that enhance their antimicrobial effectiveness (Hao et al., 2023; Vasiliev et al., 2023). The combination of silver and copper imparts improved physicochemical stability and facilitates a controlled release of metal ions, thereby intensifying microbial membrane disruption and intracellular damage (Fan et al., 2021). These nanoparticles exhibit display greater bactericidal activity effectiveness than their monometallic counterparts, targeting a broad spectrum of pathogenic microorganisms. Furthermore, the dual-metal composition may mitigate the development of microbial resistance, underscoring their potential application in food preservation, medical devices, and environmental disinfection (Hao et al., 2023). Although green synthesis of AgNPs and CuNPs has been reported in several studies, investigations exploring the green synthesis of bimetallic Ag-Cu BMNPs remain comparatively limited, especially in the context of foodborne pathogens.

The present study aims to evaluate and compare the antimicrobial effectiveness of silver, copper, and bimetallic silver-copper nanoparticles against selected foodborne pathogens (*Salmonella* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus*) with the goal of elucidating their potential as effective antimicrobial agents.

## Materials and Methods

### Materials

Silver nitrate (AgNO<sub>3</sub>), copper sulfate pentahydrate (CuSO<sub>4</sub>·5H<sub>2</sub>O), Mueller-Hinton agar, Tryptic Soy Broth, Agar-agar was purchased from Merck (Darmstadt, Germany). *S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 19115) and *S. Typhimurium* (ATCC 14028) were provided by the Food Hygiene and Technology Department at Burdur Mehmet Akif Ersoy University. Pomegranate (*Punica granatum*) peels were obtained from local suppliers in Isparta, Türkiye.

## Methods

### Preparation of plant extracts

Pomegranate peel was ground using a laboratory grinder, and the resulting powder was used as the raw material for the extraction process, which was carried out according to Asif et al. (2022) with slight alterations. Extraction was carried out with distilled water at a solid-to-liquid ratio of 1:10 (w/v), under continuous magnetic stirring (60 °C, 2 h). The mixture was subsequently centrifuged (4000 rpm, 10 min), passed through Whatman No. 1 filter paper, and the resulting supernatant was stored at -80 °C until further analysis.

### Synthesis and preparation of monometallic and bimetallic nanoparticles

Silver and copper nanoparticles were produced via a green approach employing plant extract, which served both as a reducing and stabilizing agent. Stock solutions of silver nitrate (AgNO<sub>3</sub>) and copper sulfate pentahydrate (CuSO<sub>4</sub>·5H<sub>2</sub>O) were prepared at concentrations of 0.025, 0.05, and 0.1 M. To obtain Ag-Cu BMNPs, aqueous solutions of 0.05 M AgNO<sub>3</sub> and 0.10 M CuSO<sub>4</sub>·5H<sub>2</sub>O were combined at Ag/Cu ratios of 1:1, 1:2, and 2:1 (v/v). Plant extract (1 mL per 10 mL of salt solution) was added dropwise manner under magnetic agitation (300 rpm) at 75 °C. The sealed mixtures were stirred continuously for 60 min and then incubated in darkness for 18 h to facilitate complete reduction. Successful nanoparticles formation was confirmed by the characteristic color change observed in the solutions.

### Determination of antimicrobial activity

The antimicrobial potential of the samples was evaluated through both disk diffusion and agar well diffusion assays.

### Disc diffusion test

Fresh bacterial cultures grown for 18 h were standardized to 0.5 McFarland turbidity and spread evenly on Mueller Hinton Agar plates with a sterile swab. Sterile paper discs (6 mm, Whatman™ 2017-006) were loaded with 30 µL of either monometallic or bimetallic nanoparticles suspensions and placed onto the inoculated agar. After being incubated at 37 °C for 24 h, the inhibition zone diameters were recorded (Ngamsurach & Praipipat, 2022).

### Well diffusion test

Fresh bacterial cultures (18 h) were prepared to achieve to a turbidity equivalent to the 0.5 McFarland standard, and the inoculum was uniformly spread onto Tryptic Soy Agar. Agar wells (8 mm in diameter) were aseptically prepared using a sterile cork borer. Each well was loaded with 100 µL of the nanoparticle suspension, and the inoculated plates were subjected to 24 h incubation at 37 °C. Following incubation, the inhibition zone diameters were determined using a ruler with millimeter precision (Balouiri et al., 2016).

### Statistical Analysis

All experimental data were analyzed to one-way ANOVA implemented in SPSS software program (version 20.0; SPSS Inc., Chicago, IL, USA). Each assay was conducted in duplicate. When statistical significance was observed ( $p < 0.05$ ), Duncan's Multiple Range Test was employed to assess significant differences among means.

## Results and Discussion

The antimicrobial activity of silver and copper nanoparticles synthesized at different concentrations (0.025, 0.05, and 0.1 M) was evaluated against *S. Typhimurium*, *L. monocytogenes*, and *S. aureus* using the disk and well diffusion method. The inhibition zone diameters are presented in Table 1. Silver nanoparticles exhibited considerable antibacterial activity against all tested strains, with inhibition zones ranging from 16.00 to 21.33 mm. A concentration-dependent effect was observed, particularly for *S. aureus*, where the 0.05 M AgNPs produced the largest inhibition zone ( $p < 0.05$ ). *L. monocytogenes* showed the highest sensitivity at 0.1 M and 0.05M AgNPs ( $p < 0.05$ ), while *S. Typhimurium* displayed relatively similar inhibition zones across all concentrations, and no significant differences were observed. In contrast, copper nanoparticles demonstrated lower antibacterial activity compared to AgNPs. Inhibition zones ranged from 8.00 to 10.33 mm, and *L. monocytogenes* exhibited no inhibition zone (NI) at any tested concentration. No detectable antimicrobial activity against *L. monocytogenes* may be attributed to the limited diffusion and ion release capacity of CuNPs under the tested conditions, as well as possible aggregation of the particles within the agar matrix. Against *S. Typhimurium*, the inhibition zone was greatest ( $p < 0.05$ ) at 0.05 M CuNPs ( $10.33 \pm 0.58$  mm), followed closely by 0.1 M ( $10.00 \pm 1.00$  mm), with no significant difference between these two concentrations. However, at 0.025 M, the inhibition zone decreased notably ( $8.00 \pm 1.00$  mm), indicating a significant reduction in antibacterial efficiency at the lowest concentration tested ( $p < 0.05$ ). For *S. aureus*, no significant differences in inhibition zones were observed among the tested concentrations of CuNPs. Well diffusion assay results for silver and copper nanoparticles (0.025, 0.05 and 0.1 M) against *S. Typhimurium*, *L. monocytogenes*, and *S. aureus* are presented in Table 2. Silver nanoparticles exhibited moderate antibacterial activity across all tested microorganisms, with inhibition zones ranging from 10.00 to 13.33 mm. AgNP concentration had no significant effect on the inhibition zones of all tested pathogens. In contrast, copper nanoparticles demonstrated weaker antibacterial activity compared to AgNPs, with inhibition zones ranging from 9.00 to 12.33 mm. The antibacterial activity of CuNPs against *S. Typhimurium* was similar at 0.1 M and 0.05 M, but significantly different at 0.025 M ( $p < 0.05$ ). For *L. monocytogenes*, the antibacterial activity of CuNPs decreased markedly with concentration ( $p < 0.05$ ). The highest inhibition was observed at 0.1 M, while smaller zones were recorded at 0.05 M and 0.025 M ( $p < 0.05$ ).

CuNPs showed the strongest antibacterial effect against *S. aureus* at 0.1 M, while activity declined at 0.05 M ( $p < 0.05$ ). At the lowest concentration (0.025 M), no inhibition was observed. Similar trends have also been reported in previous studies, where silver nanoparticles consistently demonstrated higher antimicrobial activity than copper nanoparticles, largely due to their stronger capability of generating ROS and interacting with microbial cell membranes of microorganisms (Gong et al., 2007; Rai et al., 2009). Ruparelia et al. (2008) reported that silver nanoparticles consistently produced larger inhibition zones than copper nanoparticles against a range of bacterial strains, including *Escherichia coli* and *S. aureus*, thereby confirming the superior antimicrobial efficiency of AgNPs. In addition, the enhanced antimicrobial efficiency of AgNPs has been attributed to the higher release of  $Ag^+$  ions, which disrupt multiple cellular processes including protein synthesis, enzymatic function, and DNA replication (Pal et al., 2007). Several studies have reported a clear dose-dependent antimicrobial effect of silver nanoparticles. For instance, Kora and Arunachalam (2011) demonstrated that AgNPs concentration increased, the inhibition of *Pseudomonas aeruginosa* growth intensified, confirmed via well diffusion assays and MIC determinations. Pazos-Ortiz et al. (2017) reported that the antibacterial effect of AgNP-incorporated polycaprolactone nanofibers against both Gram-positive and Gram-negative bacteria was strongly correlated with the applied dose, highlighting a clear dose-dependent response. These findings are in agreement with our results showing that AgNPs and CuNPs exhibit concentration-dependent antimicrobial effects, thereby justifying the selection of specific optimal concentrations for both nanoparticles types.

Based on the antibacterial activity results, 0.05 M was selected as the optimal concentration for AgNPs, as it produced the largest inhibition zone, particularly against *S. aureus*, without a significant increase at higher concentrations ( $p < 0.05$ ). In the case of CuNPs, 0.1 M was selected since it exhibited the strongest inhibitory effect among the tested concentrations, with significantly reduced activity at lower levels ( $p < 0.05$ ). With the selected concentrations (0.05 M AgNPs and 0.1 M CuNPs), Ag-Cu bimetallic nanoparticles were synthesized at different ratios (1:1, 1:2, and 2:1, v/v). The antibacterial activities of BMNPs were subsequently evaluated by both disk and well diffusion methods, and the results are presented in Tables 3 and 4, respectively.

Table 1. Zone of inhibition (mm) of silver and copper nanoparticles at different concentrations (Disk diffusion assay)

Concentration of Ag NPs (M)	Microorganism and zone diameters (mm)		
	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>
0.1	16.00 ± 1.00 <sup>a</sup>	19.00 ± 1.00 <sup>a</sup>	19.00 ± 0.00 <sup>b</sup>
0.05	17.00 ± 0.00 <sup>a</sup>	18.00 ± 0.00 <sup>ab</sup>	21.33 <sup>a</sup> ± 1.16 <sup>a</sup>
0.025	16.33 ± 0.58 <sup>a</sup>	17.33 ± 0.58 <sup>b</sup>	19.67 ± 0.58 <sup>b</sup>
Concentration of Cu NPs (M)	Microorganism and zone diameters (mm)		
	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>
0.1	10.00 ± 1.00 <sup>a</sup>	NI	9.67 ± 0.58 <sup>a</sup>
0.05	10.33 ± 0.58 <sup>a</sup>	NI	9.33 ± 0.58 <sup>a</sup>
0.025	8.00 ± 1.00 <sup>b</sup>	NI	9.00 ± 0.00 <sup>a</sup>

Values are mean ± SD; different superscript letters within a column denote significant differences. NI: No Inhibition (no inhibition zone observed).

Table 2. Zone of inhibition (mm) of silver and copper nanoparticles at different concentrations (Well diffusion assay)

Concentration of Ag NPs (M)	Microorganism and zone diameters (mm)		
	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>
0.1	11.67 ± 0.58 <sup>a</sup>	13.33 ± 0.58 <sup>a</sup>	10.00 ± 0.00 <sup>a</sup>
0.05	12.00 ± 0.00 <sup>a</sup>	12.67 ± 1.16 <sup>a</sup>	12.33 ± 3.22 <sup>a</sup>
0.025	12.00 ± 0.00 <sup>a</sup>	11.67 ± 0.58 <sup>a</sup>	10.33 ± 0.58 <sup>a</sup>
Concentration of Cu NPs (M)	Microorganism and zone diameters (mm)		
	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>
0.1	10.33 ± 0.58 <sup>a</sup>	12.33 ± 0.58 <sup>a</sup>	11.67 ± 0.58 <sup>a</sup>
0.05	10.00 ± 0.00 <sup>a</sup>	10.00 ± 0.00 <sup>b</sup>	11.00 ± 0.00 <sup>b</sup>
0.025	9.00 ± 0.00 <sup>b</sup>	9.00 ± 0.00 <sup>c</sup>	NI

Values are mean ± SD; different superscript letters within a column denote significant differences. NI: No Inhibition (no inhibition zone observed).

Table 3. Zone of inhibition (mm) of Ag–Cu bimetallic nanoparticles at different ratios (Disk diffusion assay)

Ag–Cu BMNPs (Ag/Cu, v/v)	Microorganism and zone diameters (mm)		
	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>
1:1	19.22 ± 1.20 <sup>a</sup>	19.00 ± 0.87 <sup>a</sup>	20.78 ± 1.20 <sup>a</sup>
1:2	19.44 ± 1.01 <sup>a</sup>	19.00 ± 1.00 <sup>a</sup>	20.67 ± 0.71 <sup>a</sup>
2:1	17.78 ± 0.83 <sup>b</sup>	19.11 ± 0.60 <sup>a</sup>	21.00 ± 1.00 <sup>a</sup>

Values are mean ± SD; different superscript letters within a column denote significant differences.

Table 4. Zone of inhibition (mm) of Ag–Cu bimetallic nanoparticles at different ratios (Well diffusion assay)

Ag–Cu BMNPs (Ag/Cu, v/v)	Microorganism and zone diameters (mm)		
	<i>S. Typhimurium</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>
1:1	12.78 ± 0.67 <sup>a</sup>	11.67 ± 0.87 <sup>a</sup>	13.33 ± 1.41 <sup>a</sup>
1:2	13.11 ± 0.78 <sup>a</sup>	11.78 ± 0.67 <sup>a</sup>	11.44 ± 0.53 <sup>b</sup>
2:1	13.33 ± 0.71 <sup>a</sup>	11.33 ± 1.22 <sup>a</sup>	12.33 ± 0.87 <sup>b</sup>

Values are mean ± SD; different superscript letters within a column denote significant differences.

All ratios showed strong inhibitory effects against the tested pathogens. For *L. monocytogenes* and *S. aureus*, no significant differences in inhibition zones were observed among the ratios, whereas for *S. Typhimurium*, the 1:1 and 1:2 ratios exhibited significantly greater antibacterial activity ( $p < 0.05$ ) compared to the 2:1 ratio (Table 3). Table 4 shows that no significant differences were observed among the tested ratios for *S. Typhimurium* and *L. monocytogenes*, as the inhibition zones remained relatively similar. In contrast, for *S. aureus*, the 1:1 ratio produced the largest inhibition zone, whereas the 1:2 and 2:1 ratios exhibited significantly smaller zones ( $p < 0.05$ ). These findings indicated that the antibacterial efficiency of Ag–Cu BMNPs is influenced by the Ag/Cu ratio in a strain-dependent manner, with *S. aureus* and *S. Typhimurium* showing higher sensitivity to compositional variations ( $p < 0.05$ ), while *L. monocytogenes* was less affected. This strain-dependent behavior is consistent with previous studies demonstrating that Gram-positive and Gram-negative bacteria differ in their susceptibility to metal-based nanoparticles due to variations in cell membrane structure and permeability characteristics (Elechiguerra et al., 2005; Slavin et al., 2017). However, although cell wall structure associated with Gram classification may partly explain these differences, the present findings suggest that additional determinants beyond Gram status are involved. Specifically, the relatively lower susceptibility of *L. monocytogenes*, despite its Gram positive cell structure, suggests that strain specific physiological traits, membrane composition (such as teichoic acids and membrane proteins) and oxidative stress response systems may play a more critical role in modulating the antibacterial activity of Ag–Cu BMNPs (Fan et al., 2021). This indicates that

antimicrobial responses depend not only on Gram classification but also on species specific biochemical and structural characteristics that affect metal ion tolerance, surface interactions, and intracellular defense mechanisms. Additionally, the enhanced antimicrobial efficiency observed for Ag–Cu bimetallic nanoparticles compared to their monometallic counterparts suggests a synergistic interaction between silver and copper components. Recent studies have shown that the synergistic antimicrobial activity of Ag–Cu bimetallic nanoparticles is primarily attributed to the combined effects of enhanced ion release and elevated generation of ROS (Le et al., 2024).

Similar observations have been stated in previously conducted studies, where Ag–Cu bimetallic nanoparticles demonstrated significantly higher antibacterial activity than individual Ag or Cu nanoparticles due to synergistic effects (Gulam Mohammed et al., 2014; Hao et al., 2023). Similarly, a recent study confirmed that Ag–Cu nanoalloys exhibited significantly higher microbicidal activity than monometallic forms, likely due to optimized ion release kinetics and structural advantages that favor broad-spectrum bacterial inhibition (Videira et al., 2024).

## Conclusion

The present study demonstrated that both silver and copper nanoparticles exhibited antibacterial activity against *S. Typhimurium*, *L. monocytogenes*, and *S. aureus*, though with distinct concentration-dependent effects. Silver nanoparticles showed superior antimicrobial efficacy compared to copper nanoparticles, producing inhibition zones up to 21.33 mm in *S. aureus*, 19 mm in *L. monocytogenes*, and 17 mm in *S. Typhimurium*. The most

effective concentration of Ag NPs was 0.05 M, particularly against *S. aureus* ( $21.33 \pm 1.16$  mm), while the lower concentration (0.025 M) produced smaller inhibition zones (e.g.,  $19.67 \pm 0.58$  mm). In contrast, Cu NPs showed limited activity, with maximum inhibition zones of  $10.33 \pm 0.58$  mm in *S. Typhimurium* and  $9.67 \pm 0.58$  mm in *S. aureus*, and no detectable inhibition against *L. monocytogenes* in the disk diffusion assay. The results indicated that the antimicrobial efficiency of Ag–Cu BMNPs generally depended on their Ag/Cu ratio. It was determined that the 1:1 formulation was the most effective ratio particularly against *S. aureus* and *S. Typhimurium*. Compared to monometallic nanoparticles, the Ag–Cu BMNPs formulation exhibited stronger antibacterial activity which may be the results of superior capability of Ag–Cu BMNPs for synergistic ion release, ROS generation and improved interactions with the bacterial cell surface. The results highlighted the potential of Ag–Cu BMNPs as promising candidates for developing effective antimicrobial agents, providing an alternative approach for controlling multidrug-resistant pathogens. Thus, these nanoparticles could be utilized in food preservation systems, such as active packaging materials, antimicrobial coatings or edible films to inhibit microbial growth and extend the shelf life.

## Declarations

### Author Contribution Statement

Melis YILDIZ: Data collection, analysis, and preparation of the original draft.

Birol KILIÇ: Project administration, supervision, conceptualization, methodology, review and editing

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### Conflict of Interest

The authors confirm the absence of any conflicts of interest relevant to this research.

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