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# Eco-Friendly Synthesis of Silver Nanoparticles Using NARC-G1 Garlic and Their Antimicrobial Potential

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## Abstract

The increasing prevalence of antimicrobial resistance (AMR) has intensified the need for alternative antimicrobial agents. Green synthesis of silver nanoparticles (AgNPs) using plant-derived materials offers a sustainable approach to developing nanomaterials with antimicrobial activity. This study aimed to synthesize AgNPs using NARC-G1 garlic (Allium sativum) extract and evaluate their physicochemical characteristics, hemolytic and antioxidant properties, and antibacterial activity against standard and multidrug-resistant (MDR) bacterial strains. G1-AgNPs were synthesized using aqueous NARC-G1 garlic extract and 10 mM silver nitrate ($AgNO_3$) under varying pH, temperature, and incubation conditions. The synthesized nanoparticles were characterized using UV–visible spectroscopy, Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and particle-size analysis. Hemolytic activity was evaluated using human erythrocytes, antioxidant activity was assessed using the DPPH radical-scavenging assay, and antibacterial activity was determined using agar well diffusion and broth dilution assays against American Type Culture Collection (ATCC) and MDR bacterial strains. Optimal synthesis was achieved at pH 10 and 40 °C after 7–8 h, with a characteristic surface plasmon resonance peak at 420 nm. FTIR analysis indicated the involvement of hydroxyl, carbonyl, amine, and sulfur-containing functional groups in nanoparticle formation and stabilization. XRD confirmed a crystalline face-centered cubic structure, while particle-size analysis showed prominent populations of approximately 0.8 and 10–15 nm. Hemolytic activity increased with concentration, ranging from 4% at 7.5 µg/mL to 51% at 250 µg/mL. DPPH radical-scavenging activity reached 48% at 250 µg/mL. G1-AgNPs exhibited concentration-dependent antibacterial activity against both ATCC and MDR strains, with inhibition of up to 97% against Proteus spp. and 96% against Salmonella spp. at 125 µg/mL. NARC-G1 garlic-mediated AgNPs demonstrated successful green synthesis, defined physicochemical characteristics, antioxidant activity, and broad-spectrum antibacterial effects against MDR bacteria. The concentration-dependent hemolytic response indicates that biological activity should be evaluated alongside safety parameters when considering their potential antimicrobial applications.

**Keywords**: Antimicrobial resistance, NARC G1 Garlic, Silver nanoparticles, American type culture collection (ATCC) strains, Multidrug resistance strains

### 1. Introduction

Microbial infections are major health concern worldwide. When pathogenic microorganisms such as bacteria, fungi, parasites and viruses invade and multiply inside the host cell, they cause microbial infections. These microbes enter the body through various routes. As a result of these infections morbidity and mortality rate increase particularly in context of pandemics and epidemics (1). Septicemia is an acute medical condition where bacteria in the blood circulatory system amplified and dysregulated immune system in patient. Bacterial infections in urinary tract, lungs and abdominal cavities are the primary infection of bloodstream and the most common infection sites which allow

entry of bacteria in circulatory system (2). Resistance describes the ability of microbes to adopt and survive the effect of antimicrobial treatment failures, prolonged infections, more severe disease outcomes, higher rates of deaths and more hospital admissions are some of the public health impacts. Antimicrobial resistance is considered as one of the largest global health problems in the 21st century (3). Antimicrobial resistance occurs when microorganisms can grow in the presence of the concentration of antimicrobials that inhibit other members of the same species. Resistance can be caused by direct transfer of genes or mutation of genes encoded for resistance mechanism (4). Antimicrobial resistance or AMR has significant economic costs beyond its impact on mortality and disabilities. Among LMIC after India and China, Pakistan is the 3rd largest consumer of antibiotics. Between 2000 to 2015, in India antibiotic intake increases from 3.2 billion to 6.5 billion defined dose daily, growth rate is 103%, in China it increases from 2.3 billion to 4.2 billion, growth rate is 79% DDD and in Pakistan it increases from 800 million to 1.3 billion with growth rate of 65% DDD (5). In Pakistan unnecessary use of antibiotics leads to antibiotic resistance in some novel bacterial strains. However, in Pakistan several factors are involved in causing higher rate of drug resistance that is expected.

Several alternative approaches are employed to overcome the burden of antimicrobial resistance including the development of new antibiotics, combinational therapies, bacteriophages therapy, antimicrobial peptides, CRISPR guide RNAs, fecal microbial transplant and nanoparticles. The significance of these alternative approaches in the fight against antimicrobial resistance and the requirement for continuous research to make them in clinical settings (6). Silver nanoparticles have emerged as an effective alternative combating against different microbes, because of their unique characteristics and having ability to prevent MDR bacterial strains from growing that become resistant to present antibiotics, making them highly effective for eliminating these bacteria (7). Green synthesized nanoparticles offer a promising way to overcome the drawbacks of commercial antibiotics because they exhibit strong antimicrobial activity against a range of pathogenic microorganisms (8). Among various nanoparticles, silver nanoparticles are particularly effective against bacterial infections due of their high surface to volume ratios and offering unique crystallographic surface structure (9). Garlic is an aromatic herbaceous plant, having scientific name Allium sativum belongs to the family Alliaceae. It is one of the oldest cultivated plants, originally native to Central Asia, and is now growing all over the world for its economic, pharmaceutical and nutritional value. Garlic contains a variety of volatile and nonvolatile compounds that contribute to its bioactive and therapeutic properties, such as antibacterial, antifungal, antioxidant, anti-fungal and anti-inflammatory properties (10). A new garlic variety known as NARC-G1 garlic was recently introduced by National Agricultural Research Centre (NARC) in Islamabad, Pakistan. Previous research has concentrated larger on the genetic, agronomic and botanical characteristics of the newly created NARC-G1 cultivar. Using HPLC scientists recently analyzed the differences in allicin content between NARC-G1 garlic and traditional garlic varieties. The allicin content of NARC G1 Garlic was $4.82 \pm 0.001$ (mg/g) while the concentrations of allicin in other varieties of garlic such as Desi garlic $2.934 \pm 0.001$, Italian garlic $1.813 \pm 0.011$ and Gulabi Chinese have $0.011 \pm 0.0006$ (mg/g) (11). Due to the rapid emergence of antimicrobial resistance in pathogenic microorganisms, there is an urgent need for the discovery and development of new antimicrobials from natural sources to overcome the declining effect of conventional therapies. In this research our study aims to develop an alternative approach of treating microbial infections by utilizing silver nanoparticles synthesized by NARC G1 Garlic extract.

## 2. Materials and Methods

### 2.1 Sample collection

Fresh and mature bulbs of Allium sativum (NARC G1 garlic variety) were collected from National Agricultural Research Centre (NARC), Islamabad, Pakistan. This particular variety was selected because of its high phytochemical content, including sulfur containing compounds such as allicin that play an essential role for stabilizing and reduction of silver nanoparticles (12). To ensure garlic bulbs sterility and removal of microbial contaminations, garlic bulbs were thoroughly washed with tap water and distilled water (13). The use of NARC-G1 garlic plant material in this study complied with all relevant institutional, national, and international guidelines and legislation governing the collection, handling, and use of plant materials. The plant material was obtained from the National Agricultural Research Centre Islamabad, Pakistan, and was used for research purposes in accordance with the applicable institutional and national requirements.

### 2.2 Preparation of NARC G1 Garlic extract

The clean cloves were peeled and ground into fine paste by using sterilized mortar and pestle. Garlic paste measured 10g on weighing balance, and 100ml of distilled water in measuring cylinder the resulting mixture was subjected to the boiling method. The mixture was filtered using Whatman filter paper to remove solid residues, resulting in a clear, pale yellow aqueous extract.

### 2.3 Green Synthesis of Silver Nanoparticles

For the green synthesis of silver nanoparticles 50 ml of garlic extract and 50ml of 10mM $AgNO_3$ solution was mixed. Optimize the conditions and incubate the solution at different temperatures of 30°C, 40°C, 50°C, 60°C and 70°C at different pH 7,9,10,12 using NaOH (14) for different time duration. Observed the change in color of reaction solution from light yellow to dark brown which indicates the synthesis of silver nanoparticles.

### 2.4 Characterization of Green Synthesized Silver Nanoparticles

#### 2.4.1 UV-Visible Spectroscopy

The synthesis of metallic nanoparticles from its specific salt results in characteristics peak with significant absorption in a visible spectrum (15). SRP peak in the range of wavelength of 200-800 nm is suitable for identifying nanoparticles size in the range of 2-100 nm (16).

#### 2.4.2 Fourier Transform Infrared Spectroscopy

FTIR was utilized to evaluate various functional groups involved in capping and stabilization of green synthesized silver nanoparticles and examined the involvement of biomolecules in nanoparticles formation. Pallet was loaded into sample holder and scanned in the range of 4000-500$cm^{-1}$. FTIR consists of passing infrared photons through the sample, some of the rays were absorbed by the sample and remaining was passed through it. The spectra obtained show the absorption and transmission of the sample material (17, 18).

### 2.4.3 X-Ray Diffraction Analysis

XRD pattern indicates the presence and formation of crystalline nanoparticles. By using the Bragg Reflection Law, calculate the width of Bragg reflection by using the formula, the Debye-Scherrer equation which is applied to get the particle size from the XRD data, according to the equation: $d=K\lambda/\beta \cos \theta$, where d is the particle size (nm), K is the Scherrer constant, λ is the X-ray wavelength, β is the full width half maximum and θ is the diffraction angle (half of Bragg angle) its correspond to the lattice plane (19).

### 2.4.4 Particle Size Analysis

The particle size analysis of green synthesized silver nanoparticles was analyzed using HORIBA LA-920 Laser Scattering Particle Size Distribution Analyzer. This can be explained based on the principle of Mie scattering theory, which is the basis for instrument's performance. Particle size and scattering can be analyzed by the intensity and angular distribution of scattered light.

### 2.5 Hemolytic Activity of G1-AgNPs:

Human blood was obtained as a routine normal blood sample from Diagnostic Hub, Pak-Austria Fachhochschule Institute of Applied Sciences and Technology, Haripur, Pakistan. The sample was used for erythrocyte isolation and the subsequent hemolysis assay. The blood sample was handled without collecting or reporting any personally identifiable information.

Human erythrocytes were separated by centrifugation and washed with PBS. A 1500 µL volume of G1-AgNP suspension was mixed with 1500 µL of the erythrocyte suspension and incubated for 1 h at 37 °C. After incubation, intact erythrocytes were removed by centrifugation, and the absorbance of the supernatant was measured. Triton X-100 and PBS were used as positive and negative controls, respectively.The hemolytic activity of G1-AgNPs was evaluated using different concentrations of G1-AgNPs such as 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml, 15.6µg/ml and 7.5µg/ml on human erythrocytes. Human blood was centrifugated and the erythrocytes cells were washed with PBS. By taking 1500µl of G1-AgNPs and 1500µl of RBCs suspension were mixed and incubated for 1 hour at 37°C. After incubation intact RBCs were removed by the centrifugation of sample at 8,000 rpm for 15 minutes. The supernatant diluted with PBS ten times and absorbance was recorded using UV Visible spectrophotometer at 540nm wavelength (20). Triton X 100 was taken as positive control and PBS as a negative control.

(%) hemolysis = (control OD – sample OD)/ sample OD×100

### 2.6 DPPH (2,2-Diphenyl1-1-picrylhygrazyl) Radical scavenging Assay

G1-AgNPs free radical activity was evaluated using DPPH assay. DPPH reagent was prepared by adding 1mg of DPPH powder in 25ml of dimethyl sulfoxide and different concentrations of G1-AgNPs were prepared (250µg/ml, 125µg/ml, 62.5µg/ml, 31.5µg/ml, 15µg/ml and 7.5µg/ml). The procedure consists of taking 2.85ml of DPPH reagent and adding 0.15ml of each sample of G1-AgNPs. The reaction solution was incubated at room temperature for 40 minutes in dark condition. After 40 minutes UV-Visible spectrophotometer was used to take readings of reaction mixture, and absorbance of the solution was measured at 515nm (21).

(%) inhibition of free radical = (Control OD - Sample OD)/ Control OD×100

### 2.7 Collection and Culturing of Bacterial strains

ATCC bacterial strains obtained from Qarshi Research International (Pvt) Ltd and Multidrug resistant bacterial strains were obtained from a diagnostic laboratory of local hospitals of Haripur, KPK. Both strains were cultured on general-purpose media and selective media. For morphological traits discrete colonies were observed. For identification, bacteria grown on selective and differential media. *Escherichia coli, Proteus* species, *Salmonella* species, *Klebsiella species* were grown on *MacConkey agar* and *Serratia marcescens* on CLED agar and *Pseudomonas aeruginosa* on Xylose Lysine Deoxycholate (XLD) agar, *Methicillin Resistant Staphylococcus aureus* on Blood Agar and *Staphylococcus capitis* on Mannitol salt agar.

#### 2.7.1 Gram Staining

Gram staining was performed to differentiate the bacterial isolates due to their cell walls differentiation, which allows preliminary classification of Gram-positive and Gram-negative bacteria. Added one drop of distilled water in the center of slide and picked a single colony of bacteria to prepare smear. Allow the slide to dry and add crystal violet for 30 seconds, rinse the slide with distilled water and apply gram iodine for 60 seconds which generates crystal violet complex and washed with distilled water. 95% ethanol was added on slide as a decolorizer for 10 seconds to prevent over decolorizer immediately rinse the slides with distilled water. Safranin counterstain was added for one minute on slide and rinsed with distilled water. Finally microscopic examinations were performed at 100x using immersion oil (22).

#### 2.7.2 Biochemical Testing

Urease test was evaluated by using Christensen's Urea Agar slants. Urease test identifies that organisms hydrolyze urea to produce carbon dioxide and ammonia (23). Simmons Citrate agar was used to identify the ability of citrate as a source of carbon and energy. Picked a single colony of bacteria streaked and stabbed across the entire surface of slant (24). The indole test was performed to determine the potential of bacterial isolates to degrade tryptophan and produce indole. Picked a single colony of bacteria and added to tryptone broth and incubated at 37°C for 18-48 hours. The catalase test was performed to indicate the presence of catalase enzyme produced by bacteria, which catalyze the breakdown of hydrogen peroxide into water and oxygen (25). The coagulase test was performed to identify the coagulase enzyme in bacteria (26). The oxidase test was performed to determine the presence of cytochrome oxidase enzyme in the chosen MDR strains (27).

### 2.8 Antibacterial Assays of G1-AgNPs

#### 2.8.1 Agar Well Diffusion Method

The antimicrobial activity of G1-AgNPs was evaluated using agar well diffusion method against ATCC bacterial and multidrug resistant bacterial strains. Bacterial suspension in nutrient broth was taken and adjusted to 0.5 McFarland turbidity standard by measuring OD600 on UV-Visible spectrophotometry in the range of 0.08-0.1, pick 100µl of inoculum and added on Muller Hinton Agar plate prepare uniform lawn. A sterilized cork borer was used to create a

well in agar medium plates, pour 100µl of G1-AgNPs stock solution added in well carefully and standard antibiotic employed as a positive control and incubated at 37°C for 24 hours and after 24 hours zone of inhibition was measured (28).

### 2.8.2 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of G1-AgNPs was determined by the lowest concentration that completely inhibits prominent growth of microorganism on agar surface to calculate MIC (29). In 96 well plate add 100µl of Muller Hinton Broth in all wells and 100µl of G1-AgNPs stock and form 2-fold serial dilutions and finally add 100µl of bacterial suspension in each well. Muller Hinton Broth as a negative control and bacterial suspension as a positive control. Incubated the plate at 37°C for 24 hours and the absorption was recorded at 600 nm.

## 3. Results

### 3.1 Green Synthesis of Silver Nanoparticles

NARC G1 garlic extract was used to successfully synthesize silver nanoparticles using 10mM $AgNO_3$ solution. The solution color change was observed from yellowish to dark brown at pH 10, at 40°C for 7-8 hours which indicated the synthesis of silver nanoparticles (30).

### 3.2 Characterization of Synthesized Silver Nanoparticles

#### 3.2.1 Characterization by UV–Vis Spectrophotometer

UV-Visible spectroscopy measures the amount of light absorbed by the sample (31). In this study, AgNPs was observed at 420nm, (Figure 1) which shows strong and distinctive absorption peak indicates that the active compound was successfully purified and present. The change in color of reaction solution indicates the reduction of silver ions, which attributes the presence of bioactive compounds in plant extract that helps to reduce silver nitrate to metallic silver, such as allicin which acts as reducing agent (32).

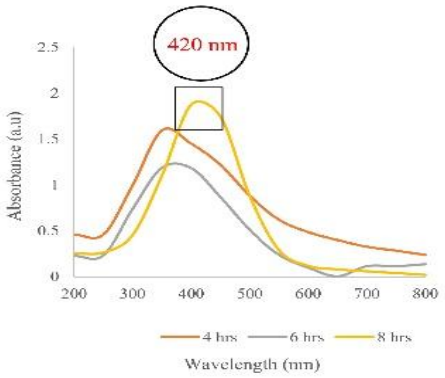



Figure 1: UV–Vis Spectrum of G1-AgNPs at 10mM concentration of AgNO3 and pH 10 displays a characteristic SPR peak at 420 nm (with earlier peaks at 350nm after 4 and 6 hours), confirming the formation of small-sized, well-dispersed silver nanoparticles.

### 3.2.2 Characterization by Fourier Transform Infrared Spectroscopy

FTIR spectrum demonstrates the functional groups associated with the green synthesis of silver nanoparticles based on NARC G1 Garlic extract having several distinct peaks, (Figure 2) which are the involvement of bioactive compounds in the synthesis and stabilization of nanoparticles. In NARC G1 Garlic extract many components are present such as flavonoids, phenolics and sulfur containing compounds which contribute to the stability and bioactivity of silver nanoparticles by bio reduction and capping of silver ions.

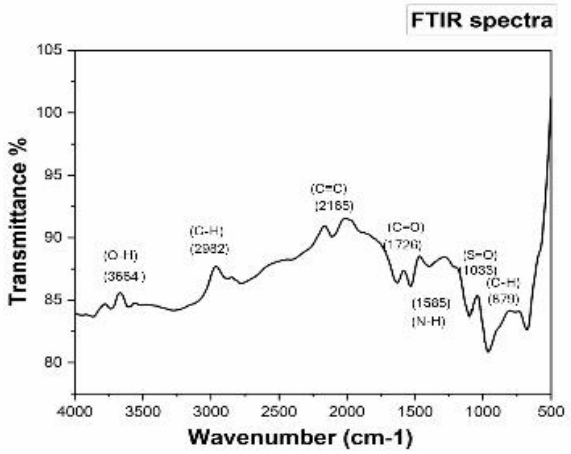



**Figure 2** FTIR analysis of NARC G1 Garlic based silver nanoparticles.

**Table 1** Functional groups and phytochemical compounds present in Garlic based silver nanoparticles.

| eaks | unctional groups | ompounds |
|---|---|---|
| 664 cm⁻¹ | -H stretching | lcohol/phenol |
| 392 cm⁻¹ | -H stretching | lkane/sulfur containi<br>ompounds |
| 165 cm⁻¹ | ≡C stretching | lkyne |
| 726 cm⁻¹ | =O stretching | ldehyde |
| 585 cm⁻¹ | -H bending | mine |
| 033 cm⁻¹ | =O stretching | ulfoxide |

### 3.2.3 Characterization by X-Ray Diffraction (XRD):

X-ray diffraction performed to evaluate the crystalline structure and phase purity of green synthesized silver nanoparticles. The crystalline structure of silver nanoparticles was recorded by diffraction pattern at angle of 2θ in the range of 20° to 80° which clearly exhibited distinct diffraction peaks. In XRD spectra diffraction planes were found at 27.19°, 32.43° and 46.34° approximately corresponding to diffraction planes (111), (200) and (220) (Figure 3). The

synthesis of pure silver nanoparticles was confirmed by these peaks, which demonstrate the characteristics of face centered cubic crystalline structure of silver nanoparticles.

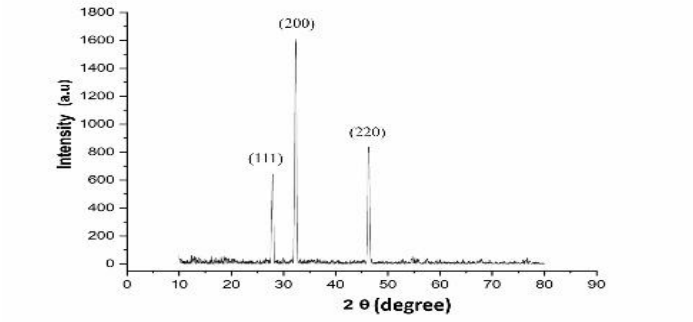



**Figure 3** XRD analysis of NARC G1 Garlic based silver nanoparticles.

**Table 2** X- Ray diffraction analysis showing crystalline size of synthesized nanoparticles.

| Angles | Planes | FWHM | Crystalline Size (nm) |
|---|---|---|---|
| 27.19° | 111 | 1.4 | 5.85nm |
| 32.434° | 200 | 1.1 | 7.52nm |
| 46.34° | 220 | 1.3 | 6.65nm |

### 3.2.4 Particle Size Analysis

G1-AgNPs shows bidirectional distribution with two prominent peaks. A population of very small nanoparticles located by the first peak at approximately 0.8nm, the second peak is broader having size of 10-15nm denoting larger particles (Figure 4). Two distinct populations of nanoparticles were synthesized by using garlic extract.

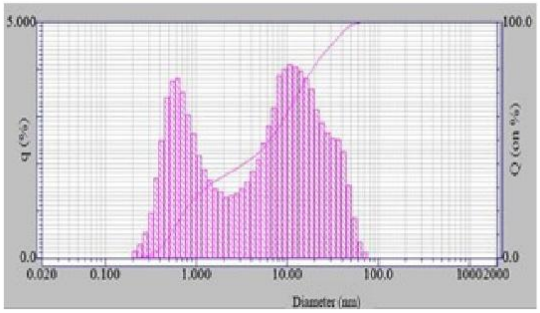



**Figure 4** The particle size distribution of G1-AgNPs, q% (distribution) and Q% (cumulative) curve shows a bimodal pattern with peaks from 0.8 nm to 100 nm.

### 3.3 Hemolytic Activity of G1-AgNPs

At the highest concentration of 250µg/ml a maximum hemolysis of 51% was observed indicate membrane disruption at higher nanoparticle doses. With the decrease in concentration of G1-AgNPs the percentage of hemolysis declined 32% at 125µg/ml, 25% at 62.5µg/ml and 18% at 31.5µg/ml. While the lowest concentration of G1-AgNPs shown moderate to weak hemolytic activity with 13% inhibition at 15µg/ml and minimum inhibition of 4% at 7.5µg/ml, indicated G1-AgNPs are less toxic at lowest concentration (Figure 5).

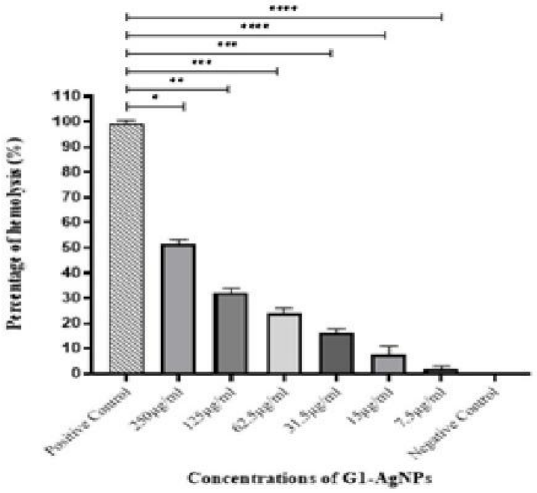



**Figure 5** Dose-dependent reduction in hemolytic activity of G1-AgNPs compared to the positive control (1% Triton X-100) and negative control (PBS) ($p < 0.001$).

### 3.4 DPPH Radical Scavenging Assay of G1-AgNPs

G1-AgNPs shown inhibition of 48% at 250µg/ml concentration which is the highest concentration and 37% at 7.5µg/ml which was lowest concentration for testing (Figure 6).

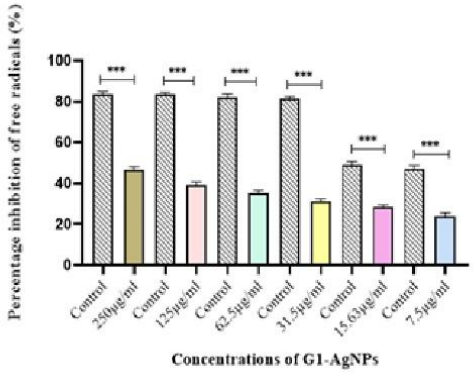



**Figure 6** Antioxidant activity of G1-AgNPs measured by DPPH assay, significant difference observed from the positive control (ascorbic acid) ($p < 0.001$).

### 3.5 Isolation and Culturing of Bacterial strains

Bacterial strains were cultured on general purpose media and differential media. After incubation, distinct colony characteristics were observed for each organism.

**Table 3** Characteristics of colonies on nutrient agar and differential agar media

| S. No | Bacterial Strains | Colony Morphology on Nutrient agar | Colony Morphology on differential agar media |
|---|---|---|---|
| 01 | Escherichia coli | Large, thick, greyish white, moist, opaque colonies | Pink colonies on MacConkey agar |
| 02 | Proteus species | Pale white colonies with a thin filmy layer of concentric circles. | Flat colorless colonies (non- lactose fermenting) on MacConkey agar |
| 03 | Pseudomonas aeruginosa | Opaque, large, flat with irregular margins, greenish colonies | Pink, flat, and rough colonies on XLD agar |
| 04 | Salmonella species | Off-white, moist, and have smooth, convex surfaces with complete margins | Colorless colonies on MacConkey agar, blue-green, translucent, and smooth colonies on CLED agar |
| 05 | Klebsiella species | Circular, dome-shaped, mucoid, translucent white colonies | Circular, convex, mucoid, light pink colored opaque colonies on MacConkey agar |
| 06 | Serratia marcescens | Red, smooth, convex, entire, and round colonies; red color due to production of pigment | Small, slightly bluish-white colonies on CLED agar |
| 07 | Methicillin resistant staphylococcus aureus | Small, circular, opaque colonies with a golden yellow pigment | Large, round, creamy white colonies,often with hemolysis on Blood agar |
| 08 | Staphylococcus capitis | Circular, cream-colored to white | Yellow and pink round, large and fermented on MSA |

#### 3.5.1 Gram Staining

Gram staining was performed on each of the retrieved bacterial isolates for the initial identification of the characteristics of bacteria. Some strains results show red/ pink color in microscopy of slides that are the characteristics of gram-negative bacteria due to the retaining of safranin counter stain. Morphology of bacteria was observed under microscope as a small rod shaped called bacilli.

**Table 4** Gram Staining of Multidrug resistant bacterial strains

| S. No | Bacterial isolates | Gram Staining |
|---|---|---|
| 01 | *Escherichia coli* | Gram Negative Rods |
| 02 | *Proteus species* | Gram Negative Rods |
| 03 | *Pseudomonas aeruginosa* | Gram Negative Rods |
| 04 | *Salmonella species* | Gram Negative Rods |
| 05 | *Klebsiella species* | Gram Negative Rods |
| 06 | *Serratia marcescens* | Gram Negative Rods |
| 07 | *Staphylococcus aureus* | Gram Positive cocci |
| 08 | *Staphylococcus capitis* | Gram Positive cocci |

### 3.5.2 Biochemical testing

**Urease test**

Color change of In Christensen's Urea Agar slants was observed in *Proteus species, Klebsiella species, Serratia marcescens* and *Methicillin Resistant Staphylococcus aureus*, from light yellow to pink and some strains including *Escherichia coli, Pseudomonas aeruginosa, Salmonella app* and *Staphylococcus capitis* conserves its original light yellow color, which indicated the absence of urease enzyme activity, result demonstrated that some bacterial strain tested was urease positive and some are negative.

**Citrate test**

Citrate tests were performed for all bacterial isolates. Among them only *Pseudomonas aeruginosa, Salmonella species, Klebsiella species, Serratia marcescens* and *Methicillin Resistant Staphylococcus aureus* shows positive citrate reaction, indicated by the visible growth and a prominent blue coloring of Simmons Citrate Agar slant. While *Escherichia coli, Proteus species,* and *Staphylococcus capitis* show negative results, no growth and retain their original natural green color.

**Indole test**

Inoculated tryptone broth was incubated and addition of Kovac's reagent in all tubes, some isolates exhibit cherry red to pink color which indicated positive indole result of *Escherichia coli, Proteus species* and *Klebsiella species*. No apparent changes in color of the reagent was observed in *Pseudomonas aeruginosa, Salmonella app, Serratia marcescens, Methicillin Resistant Staphylococcus aureus* and *Staphylococcus capitis* show negative.

**Catalase test**

On glass slide bacterial inoculum and hydrogen peroxide were added immediate formation of bubble was observed which indicate the presence of catalase enzyme. *Escherichia coli, Proteus species, Salmonella species, Klebsiella species, Serratia marcescens* and *Methicillin Resistant Staphylococcus aureus* were catalase positive while *Methicillin Resistant Staphylococcus aureus* are catalase negative.

**Coagulase test**

Coagulase test was performed for all bacterial strains, among them only *Pseudomonas aeruginosa* and *Methicillin resistant Staphylococcus aureus* exhibit positive results. The remaining strains are Coagulase negative. Positive results were observed for 3 strains show clear clumping in the test slide and clot formation confirm both bound and free coagulase enzymes are present. These results demonstrate the utility of coagulase test for differentiating coagulase positive strains.

**Oxidase test**

In the oxidase test *Pseudomonas aeruginosa* show positive results indicated by the development of dark purple color which confirms the presence of cytochrome oxidase enzyme. Remaining strains show no change in color which demonstrated oxidase negative.

### 3.6 Antibiotic Susceptibility of Clinical Resistant Bacterial Strains

Antibiotic susceptibility testing was performed against all isolated bacterial strains in which antibiotics of specific strain were used against them all strains show stronger resistance to their antibiotics shown in table 5.

**Table 5** Antibiotic susceptibility of multidrug resistance bacterial strains

| MDR Bacterial Strains | Antibiotics | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AMX | AMP | AZM | FEP | LEV | CRO | CIP | TMP | MEM | NIT | CN | IPM | P | OX | CLI |
| *E. coli* | R | | | | R | | R | | | S | | | | | |
| *Proteus spp* | | | | | R | R | R | | | | S | | | | |
| *P. aeruginosa* | | | | R | S | | R | | S | | S | S | | | |
| *Salmonella spp* | | R | R | | | S | | I | | | | | | | |
| *Klebsiella spp* | | | | | | | | | R | | | R | | | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. Marcescens* | R | | | | | | | | S | | | S | | | |
| *Staphylococcus spp* | | | | | | | | | | | | | R | R | S |

### 3.7 Antibacterial Assays of G1-AgNPs

#### 3.7.1 Agar Well Diffusion Method

**G1-AgNPs Activity Against ATCC Bacterial Strains by Agar Well Diffusion Method**

G1-AgNPs exhibited varying level of antimicrobial activity against ATCC strains, they exhibit larger zone of inhibition against *Listeria monocytogens, Listeria innocua* and *Cronobacter sakaazakii* compared to standard antibiotics. Moderate effectiveness was shown against *Enterococcus Faecalis, Salmonella tranoroa and Pseudomonas aeruginosa*, their zone of inhibition was slightly lower than standard antibiotics. However, nanoparticles showed less activity against *Escherichia coli* with smaller zone of inhibition as shown in Figure 7.

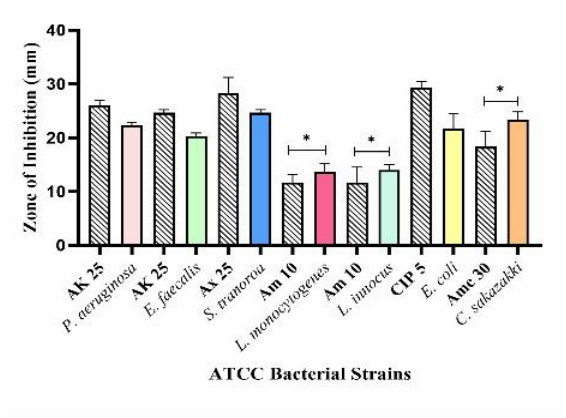



**Figure 7** Effect of G1-AgNPs (1000 µg/ml) on ATCC strains against antibiotics controls. Significant inhibition in L. monocytogenes, L. innocua, and C. sakazakii ($p < 0.05$) no significant differences (ns) for P. aeruginosa, E. faecalis, S. tranoroa, and E. coli.

**G1-AgNPs Activity Against MDR Strains by Agar Well Diffusion Method**

G1-AgNPs exhibit strong antimicrobial activity against MDR strains *Proteus species, Pseudomonas aeruginosa, Serratia marcescens, Salmonella typhi* and *Staphylococcus capitis* showed larger zone of inhibition than standard antibiotics. While *E. coli* shows 24mm ZOI a slightly larger zone but not significant. *Klebsiella species* and *MRSA* exhibit smaller zone of inhibition of G1-AgNPs as compared to their standard antibiotics (Figure 8). The results demonstrate that G1-AgNPs poses a broad spectrum of antimicrobial activity against MDR strains, where antibiotic resistance is challenging.

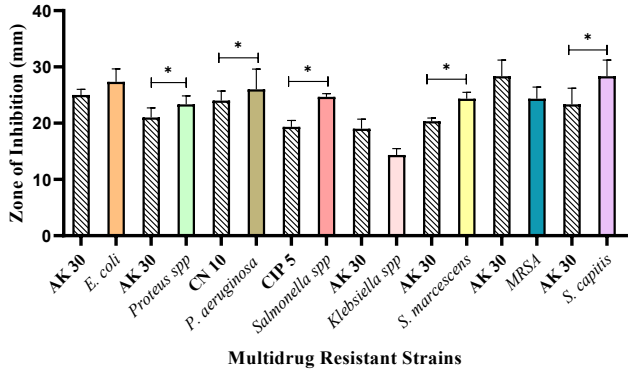



**Figure 8** Effect of G1-AgNPs (1000 µg/ml) on MDR against antibiotic controls. Significant inhibition in *Proteus spp, P. aeruginosa, Salmonella spp, S. marcescens, and S. capitis* ($p < 0.05$); *E. coli* showed a greater, but not significant, inhibition zone; no significant difference in *Klebsiella spp* and *MRSA.*

### 3.7.2 Broth Dilution Method

**G1-AgNPs Activity Against ATCC Bacterial Strains Via Broth Dilution Method**

The antibacterial activity of G1-AgNPs was assessed against ATCC bacterial strains, G1-AgNPs exhibits highest inhibition recorded from 125µg/ml to 1.95µg/ml. Each bacterial strain demonstrated concentration dependent inhibition, where the percentage of bacterial inhibition increased in correlation with the increase in concentration of G1-AgNPs. The highest level of inhibition at 125µg/ml was recorded for all bacterial strains which indicated that G1-AgNPs have significant antibacterial activity at highest level of dosage. Notably, *E. coli* exhibited strongest inhibition (95%), *Cronobacter sakazakii* demonstrates stronger sensitivity at maximum concentration achieving inhibition of (93%), *Salmonella tronoroa* (96%), *Enterococcus faecalis* (95%) and *Listeria innocua* shows (82%) inhibition. *Pseudomonas aeruginosa* and *Listeria monocytogens* exhibit comparatively lower inhibition of 91% and 78% respectively. Cronobacter sakazakii demonstrates high inhibition at all concentrations retaining over 75% inhibition at the lowest concentrations at 1.95µg/ml similarly, inhibition of *Salmonella tranoroa* demonstrates a strong dose dependent from 96% to 61% at 125µg/ml to 1.95µg/ml. However, *Listeria monocytogens* exhibited a sharp decline in inhibition at lower concentrations from 78% to 14% at 125µg/ml to 1.95µg/ml suggesting reduced sensitivity. The irregular inhibition pattern of *Pseudomonas aeruginosa* and *Listeria innocua* displayed moderate and unpredictable. A more gradual and consistent decline in activity was observed in Enterococcus faecalis from 95% to 34% at 125µg/ml to 1.95µg/ml and E. coli from 95% to 40% at 125µg/ml to 1.95µg/ml. Whereas value of *P. aeruginosa, E. faecalis, S.*

*tranoroa* and *E. coli* are 107 125µg/ml, *C. sakazakki* is 31.2µg/ml. Whereas, *L. monocytogenes* and *L. innocua* required higher concentration of G1-AgNPs for inhibition of bacteria (Figure 9).

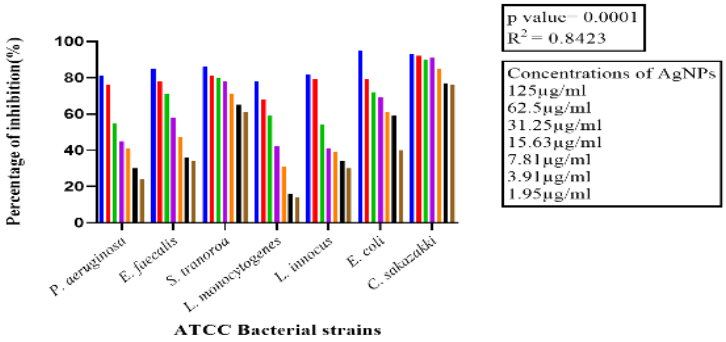



**Figure 9** Statistical analysis of all ATCC Bacterial strains show highly significant p value (****< 0.0001) and a strong correlation coefficient ($R^2 = 0.8423$), indicating that the antibacterial activity of G1-AgNPs is concentration dependent and consistent across multiple strains. These results support G1-AgNPs as an effective antimicrobial agent against a broad spectrum of bacterial pathogens.

**G1-AgNPs Activity Against MDR Bacterial Strains Via Broth Dilution Method**

The antimicrobial efficacy of G1-AgNPs was evaluated against MDR strains by using Broth dilution method. Graph clearly shows dose dependent inhibition of bacterial growth against a variety of MDR strains. G1-AgNPs exhibit excellent bacterial growth inhibition activity for most strains. Antibacterial activity of G1-AgNPs against Gram negative multidrug resistant isolates which exhibit strong and dose dependent activity of G1-AgNPs. *Proteus species* exhibit the highest susceptibility of 97% inhibition at 125µg/ml which get reduced to 26 % at 1.95µg/ml. *Salmonella species* also shows strong inhibition with 96% inhibition at 125µg/ml, and it declined to 32% at 1.95µg/ml indicating consistent antibacterial effect at lowest concentration. While *Klebsiella species, E. coli* and *Pseudomonas aeruginosa* also show strongest susceptibility, *Klebsiella species* shows 95% inhibition at 125µg/ml reduced to 25% at lowest concentration, *E. coli* exhibits 91% at 125µg/ml with activity declined to 32% at 1.95µg/ml, *Pseudomonas aeruginosa* demonstrates 90% at higher concentration and 21% at the lowest concentration. *Serratia marcescens* range from 87% to 19% inhibition with concentration, but the nanoparticles were still effective even at lowest concentration. Among the Gram-positive isolates *MRSA* and *Staphylococcus capitis* were tested, *MRSA* showed significant inhibition ranges from 82% to 21% decline with concentration of nanoparticles. *Staphylococcus capitis* show inhibition at 81% at 125µg/ml and drop to 9% at lowest concentration, which suggests that limited interaction of nanoparticles with bacterial cell wall at sub inhibitory concentration, so these 3 strains required higher concentrations for inhibition. These results suggest that G1-AgNPs have broad spectrum antimicrobial potential and it's an alternative treatment against MDR infections. The MIC value of *E. coli* and *P. aeruginosa* are 125µg/ml, *Proteus species, Salmonella species* and *Klebsiella species* are 62.5µg/ml whereas *S. marcescens, MRSA* and *S. capitis* required higher concentration of G1- AgNPs for inhibition of bacteria (Figure 10).

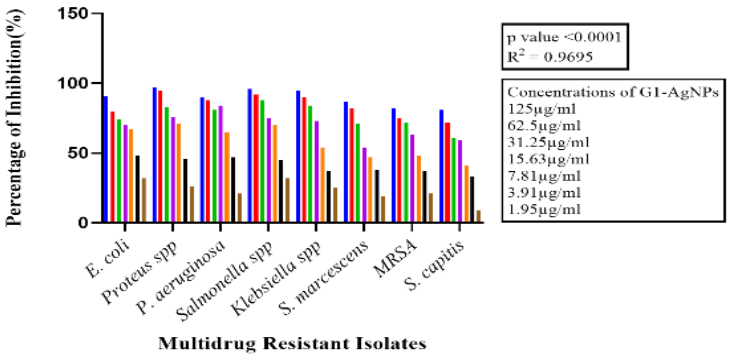



**Figure 10** Statistical analysis of all MDR strains the high R2 value (0.9608) and p value (****<0.0001) are statistically significant and demonstrate a strong linear relationship between G1-AgNPs concentration and bacterial inhibition.

## 4. Discussion

The purpose of study is to synthesize an ecofriendly and biocompatible alternative to conventional physical and chemical methods of nanoparticles synthesis by using NARC G1 Garlic (Allium Sativum) extract. The NARC G1 garlic extract was chosen because this newly developed garlic variety contains a high amount of allicin content as compared to other variety of garlic which act as capping and reducing agents in the synthesis of silver nanoparticles. The effectiveness of this synthesis process not only validates the feasibility of employing plant-based processes but also enhances the idea of using more ecofriendly methods of nanomaterial synthesis. After the successful synthesis of silver nanoparticles several characterization techniques were performed including UV-Visible spectroscopy, X-Ray diffraction, Fourier transform infrared spectroscopy and particle size analysis. UV Visible spectra exhibit the characteristics of surface plasmon resonance bands, which verify the synthesis of silver nanoparticles. FTIR confirms the presence of functional groups like phenol, alkane, alkene, alkyne, amine, aldehyde and sulfur containing compounds, which indicate their role in the synthesis of silver nanoparticles. The crystalline structure of silver nanoparticles was confirmed by XRD patterns, which exhibit characteristics peaks that resemble the face centered cubic structure of silver nanoparticles. A broad distribution profile was observed by Particle size analysis. Due to mild agglomeration or polydisperse in some particles, the size of nanoparticles varies in nanoscales, and some particles have finer dispersion. So, the influence of extract composition, preparation, conditions of nanoparticles synthesis and environmental factors are further supported by these observations.

The hemolytic assay reveals that synthesized G1-AgNPs showed minimal toxicity to human red blood cells, at lowest concentration hemolysis percentage was about 4% which indicate the appropriate use of G1-AgNPs in biomedicine because this level of hemolysis falls in the acceptable biocompatible range. The antioxidant potential of G1-AgNPs was evaluated before conducting antimicrobial testing and displayed concentration-dependent free radical scavenging activity. Maximum antioxidant activity was observed at higher doses, neutralizing oxidative stress. The biological activities of green synthesized silver nanoparticles showed promising antimicrobial activity. Nanoparticles exhibited strong inhibitory effects against ATCC bacterial strains and multidrug resistant bacterial strains. Though all the bacteria studied show susceptibility to silver nanoparticles, the level of antibacterial activity differs across bacteria. The result of study clearly indicated that NARC G1 garlic-based silver nanoparticles exhibited greater inhibitory effects against multidrug resistant bacterial strains as compared to ATCC standard strains. This rise in activity might be due to compromised defense mechanisms of MDR strains which make them more susceptible to G1-AgNPs various

modes of action including reactive oxygen species and disruption of membrane permeability. On the other hand, ATCC strains show smaller zone of inhibition due to their intact cellular response mechanism. These results suggest that G1-AgNPs have promising ability as alternative agents to target resistant infections where traditional antibiotics fail.

## Conclusion

NARC-G1 garlic-mediated synthesis provided an eco-friendly approach for producing silver nanoparticles with concentration-dependent antioxidant activity and broad-spectrum antibacterial effects, including activity against multidrug-resistant bacteria. The findings support further investigation of G1-AgNPs as a potential antimicrobial candidate, particularly through comprehensive toxicity and in vivo studies.

**List of Abbreviations:**

- **AgNPs**- Silver Nanoparticles
- **G1-AgNPs**- NARC-G1 Garlic Mediated Silver Nanoparticles
- **AMR**- Antimicrobial Resistance
- **MDR**- Multidrug Resistant
- **ATCC**- American Type Culture Collection
- **UV-Vis**- Ultraviolet–Visible Spectroscopy
- **FTIR**- Fourier Transform Infrared Spectroscopy
- **XRD**-X-Ray Diffraction
- **PBS**- Phosphate Buffered Saline
- **RBCs**- Red Blood Cells
- **DPPH**-2,2-Diphenyl-1-picrylhydrazyl
- **MIC**- Minimum Inhibitory Concentration
- **OD**- Optical Density
- **SPR**- Surface Plasmon Resonance
- **NaOH**- Sodium Hydroxide
- **$AgNO_3$**- Silver Nitrate
- **HPLC**- High Performance Liquid Chromatography
- **CLED**- Cystine Lactose Electrolyte Deficient Agar
- **XLD** - Xylose Lysine Deoxycholate Agar
- **MSA**- Mannitol Salt Agar
- **FWHM**- Full Width at Half Maximum
- **nm**- Nanometer
- **µg/ml**- Microgram per milliliter
- **rpm**- Revolutions per minute
- **pH**- Potential of Hydrogen
- **°C**- Degree Celsius

**Author contribution**

All authors made substantial contributions to the conception, study design, execution, data acquisition, analysis and interpretation; participated in drafting or critically revising the manuscript; approved the final version for publication; agreed to the journal of submission; and accept responsibility for all aspects of the work.

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**Conflicts of interest**

The authors have no conflict of interest either financial or non-financial to disclose.

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**Data availability**

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

**References**

1. Wp SE, Norhidayah M, Ar MNAJBID. Factors associated with multidrug-resistant organism (MDRO) mortality: an analysis from the national surveillance of multidrug-resistant organism, 2018-2022. 2025;25(1):60.

2. Cohen JJN. The immunopathogenesis of sepsis. 2002;420(6917):885-91.

3. Conly J, Johnston BJCJoID, Microbiology M. Where are all the new antibiotics? The new antibiotic paradox. 2005;16(3):159-60.

4. Livermore DJNRM. Can better prescribing turn the tide of resistance? 2004;2(1):73-8.

5. Qadeer A, Ullah H, Sohail M, Safi SZ, Rahim A, Saleh TA, et al. Potential application of nanotechnology in the treatment, diagnosis, and prevention of schistosomiasis. 2022;10:1013354.

6. Blaskovich MA, Cooper MAJnA, Resistance. Antibiotics re-booted—time to kick back against drug resistance. 2025;3(1):47.

7. Lee N-Y, Ko W-C, Hsueh P-RJFip. Nanoparticles in the treatment of infections caused by multidrug-resistant organisms. 2019;10:1153.

8. Singh P, Singh H, Kim YJ, Mathiyalagan R, Wang C, Yang DCJE, et al. Extracellular synthesis of silver and gold nanoparticles by Sporosarcina koreensis DC4 and their biological applications. 2016;86:75-83.

9. Sondi I, Salopek-Sondi BJJoc, science i. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. 2004;275(1):177-82.

10. Amagase H, Petesch BL, Matsuura H, Kasuga S, Itakura YJTJon. Intake of garlic and its bioactive components. 2001;131(3):955S-62S.

11. Shamshad S, Rizvi KA, Shehnaz H, Jatoi SA, Shahnaz L, Naqvi SMS, et al. Novel NARC-G1 garlic: comparative allicin quantification with morpho-biochemical & genetic profiling. 2022;84:e262697.

12. Chauhan J, Chaudhary A. Green Synthesis of Silver Nanoparticles using Garlic Extract. 2019.

13. Rai M, Yadav AJIn. Plants as potential synthesiser of precious metal nanoparticles: progress and prospects. 2013;7(3):117-24.

14. Ramzan M, Karobari MI, Heboyan A, Mohamed RN, Mustafa M, Basheer SN, et al. Synthesis of silver nanoparticles from extracts of wild ginger (Zingiber zerumbet) with antibacterial activity against selective multidrug resistant oral bacteria. 2022;27(6):2007.

15. Karikalan N, Karthik R, Chen S-M, Velmurugan M, Karuppiah CJJoC, Science I. Electrochemical properties of the acetaminophen on the screen printed carbon electrode towards the high performance practical sensor applications. 2016;483:109-17.

16. Luong NH, Long NN, Vu LV, Hai NH, Nghia P, Anh NJIjon. Metallic nanoparticles: synthesis, characterisation and application. 2011;8(3-5):227-40.

17. Rohman A, Man YCJFri. Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. 2010;43(3):886-92.

18. Zhang X-F, Liu Z-G, Shen W, Gurunathan SJIjoms. Silver nanoparticles: synthesis, characterization, properties, applications, and therapeutic approaches. 2016;17(9):1534.

19. Prathna T, Chandrasekaran N, Raichur AM, Mukherjee AJC, Biointerfaces SB. Biomimetic synthesis of silver nanoparticles by Citrus limon (lemon) aqueous extract and theoretical prediction of particle size. 2011;82(1):152-9.

20. Kokila T, Ramesh P, Geetha DJE, safety e. Biosynthesis of AgNPs using Carica Papaya peel extract and evaluation of its antioxidant and antimicrobial activities. 2016;134:467-73.

21. Tran TMK, Kirkman T, Nguyen M, Van Vuong QJH. Effects of drying on physical properties, phenolic compounds and antioxidant capacity of Robusta wet coffee pulp (Coffea canephora). 2020;6(7).

22. Moyes RB, Reynolds J, Breakwell DPJCPiM. Differential staining of bacteria: gram stain. 2009;15(1):A. 3C. 1-A. 3C. 8.

23. Brink BJAsfm. Urease test protocol. 2010:1-7.

24. MacWilliams MPJASfM. Citrate test protocol. 2009:1-7.

25. Shangari N, O'Brien PJJCpit. Catalase activity assays. 2006;27(1):7.. 1-7.. 16.

26. Katz DSJASfMLPAohwaocepp. Coagulase test protocol. 2010;3220.

27. Shields P, Cathcart LJAsfm. Oxidase test protocol. 2010;4:1-9.

28. Kim H-K, Choi S-W, Lee H-J, Lee J-H, Choi H-MJBr. Suppression of fatty acid synthase by dietary polyunsaturated fatty acids is mediated by fat itself, not by peroxidative mechanism. 2003;36(3):258-64.

29. Hossain TJJEJoM, Immunology. Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. 2024;14(2):97-115.

30. Prathna T, Raichur AM, Chandrasekaran N, Mukherjee AJAJoC. Heat mediated synthesis of silver nanoparticles using citrus limon (lemon) extract. 2013;25(Supplementary Issue):S305.

31. Mourdikoudis S, Pallares RM, Thanh NTJN. Characterization techniques for nanoparticles: comparison and complementarity upon studying nanoparticle properties. 2018;10(27):12871-934.

32. Zhou Y, Lin W, Huang J, Wang W, Gao Y, Lin L, et al. Biosynthesis of gold nanoparticles by foliar broths: roles of biocompounds and other attributes of the extracts. 2010;5(8):1351.