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RESEARCH ARTICLE

REVISED Green biosynthesized silver nanoparticles using aqueous extract of *Salix alba*: antimicrobial and cytogenetic effects on mitosis

[version 2; peer review: 2 approved]

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Abstract**Background**

Green synthesis of silver nanoparticles (AgNPs) using plant extracts has gained increasing attention as an environmentally friendly alternative to conventional chemical methods. *Salix alba* L contains bioactive phytochemicals that can act as reducing and stabilizing agents during nanoparticle synthesis. This study aimed to synthesize AgNPs using an aqueous bark extract of *S. alba* and to evaluate their antimicrobial and cytogenetic effects.

Methods

Silver nanoparticles were synthesized by mixing *S. alba* aqueous extract with silver nitrate solution. Nanoparticle formation and surface characteristics were assessed using ultraviolet visible spectroscopy, atomic force microscopy (AFM), and scanning electron microscopy (SEM). Antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* were evaluated using the agar well diffusion method. Cytogenetic effects were assessed in mice bone marrow cells by measuring the mitotic index.

Results

AFM and SEM analyses showed that AgNP size and morphology varied

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with silver nitrate concentration, with smaller and more uniform nanoparticles observed at lower concentrations. The synthesized AgNPs exhibited antimicrobial activity against all tested microorganisms, with inhibition zones increasing in a dose dependent manner. Cytogenetic analysis demonstrated a significant reduction in mitotic index in AgNP treated groups at higher concentrations, while *S. alba* extract alone did not induce genotoxic effects and maintained normal or slightly elevated mitotic index values.

Conclusions

Salix alba L mediated silver nanoparticles demonstrated antimicrobial activity and concentration dependent cytogenetic effects. These findings indicate the importance of careful dose optimization when considering biologically synthesized AgNPs for potential applications.

Keywords

Salix alba, plant extract, silver nanoparticles (NPs), antimicrobial activity, cytogenetic assay, mitotic index



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REVISED Amendments from Version 1

In this updated version of the manuscript, several revisions have been made in response to the reviewers' comments to improve the clarity, quality, and scientific rigor of the study. The Introduction section has been refined to provide clearer background information and better contextualize the significance of green biosynthesized silver nanoparticles.

The Materials and Methods section has been revised to enhance clarity and reproducibility, including more detailed descriptions of experimental procedures and conditions. In addition, terminology has been corrected to clearly distinguish between silver nitrate precursor molarity and the concentration of synthesized silver nanoparticles used in biological assays.

The Results and Discussion sections have been substantially improved. The discussion has been expanded to include deeper mechanistic interpretations supported by recent literature, and comparisons with related studies have been added where appropriate. Figures and tables have been reviewed and updated for improved clarity, labeling, and consistency.

Furthermore, language and grammatical corrections have been made throughout the manuscript to improve readability. All revisions have been clearly highlighted using Track Changes to ensure transparency and to allow readers and reviewers to easily identify the modifications made in this version.

Overall, these revisions strengthen the manuscript by addressing the reviewers' concerns and improving the presentation and interpretation of the findings.

Any further responses from the reviewers can be found at the end of the article

1. Introduction

Nanotechnology is a rapidly growing field that brings together concepts from physics, chemistry, biology, and materials science to address various challenges in medicine, industry, and the environment (Das *et al.*, 2025). Among the many types of nanomaterials, silver nanoparticles (AgNPs) have received particular attention due to their strong antimicrobial effects, catalytic properties, and promising roles in medical and diagnostic applications (Sati *et al.*, 2025). However, traditional methods for producing AgNPs often rely on harmful chemicals and require large amounts of energy, raising serious concerns about their environmental impact and long-term sustainability. As an alternative, researchers have turned to green synthesis approaches that make use of biological sources such as plant extracts, bacteria, fungi, and algae (Kirubakaran *et al.*, 2025). These eco-friendly techniques not only minimize toxicity and environmental harm but are also cost-effective and compatible with biological systems. The use of plant extracts in the eco-friendly synthesis of silver nanoparticles (AgNPs) has become increasingly favoured, primarily due to the presence of diverse bioactive compounds such as flavonoids, polyphenols, tannins, and terpenoids. These phytochemicals serve essential functions in the synthesis process, acting as natural reducing and stabilizing agents that facilitate nanoparticle formation (Shahzadi *et al.*, 2025; Aji *et al.*, 2025). A notable example is *S. alba* (commonly known as white willow), a tree native to many parts of Europe, Asia, and North America. Traditionally, species of the *Salix* have been recognized for its therapeutic potential. Among them, *S. alba* contains several important phytochemicals; such as salicin, catechins, gallic acid, and other phenolic compounds—that are responsible for its antioxidant, antimicrobial, and anti-inflammatory activities (Chaudhary *et al.*, 2025a). These naturally occurring substances not only help in transforming silver ions into nanoparticles but also act as a protective layer on the nanoparticles surface, enhancing both their stability and biological functionality (Lasmi *et al.*, 2025).

In recent years, *S. alba* has gained attention as a promising natural source for synthesizing silver nanoparticles. Studies have demonstrated that aqueous extracts derived from the bark or leaves of *S. alba* can effectively mediate the formation of silver nanoparticles, typically characterized by their small size, spherical shape, and notable surface plasmon resonance (SPR) properties (Habibi *et al.*, 2025). These green-synthesized AgNPs have exhibited significant antimicrobial effects against a broad spectrum of pathogens, including both Gram-positive and Gram-negative bacteria, as well as various fungal strains. The antimicrobial action of these nanoparticles operates through multiple pathways, such as compromising microbial cell membranes, inducing reactive oxygen species (ROS), hindering DNA replication, and inhibiting vital enzymes (Ibrahim *et al.*, 2025). Moreover, the phytochemicals present in *S. alba* extracts may enhance the biological effectiveness of the nanoparticles by forming a stabilizing organic layer, which not only boosts antimicrobial activity but may also help reduce toxicity toward human cells (Al-shattrawi, 2025).

In addition to their antimicrobial properties, the cytogenetic effects of silver nanoparticles (AgNPs) have become a growing focus of research, especially regarding their potential to cause genetic damage. One of the standard methods for evaluating genotoxicity is the micronucleus (MN) assay, which identifies small extranuclear structures that result from chromosomal fragments or entire chromosomes that are not properly integrated into daughter nuclei during cell division (Lala, 2025). The presence of micronuclei is considered a dependable marker for detecting chromosomal instability and genotoxic stress in both cell cultures and living organisms (Onoja *et al.*, 2025). As AgNPs are increasingly explored for

biomedical applications, it becomes essential to assess not just their therapeutic potential but also any cytogenetic hazards they may pose (Bentrad, 2025). Green-synthesized AgNPs, which are coated with plant-based biomolecules, may offer a safer alternative with lower genotoxicity compared to nanoparticles produced through conventional chemical methods. Nonetheless, their actual cytogenetic impact is influenced by several variables, such as particle size, surface charge, dosage, and duration of exposure (Zhang *et al.*, 2025).

2. Methods

2.1 Plant collection and pretreatment

In September 2024, leaves bark of *S. alba* plant were gathered locally from Baghdad marketplaces. The plant was identified by Dr. Ibrahim S. Al-Jubouri/College of Pharmacy /Al-Mustansiriyah University/Iraq.

2.2 Preparation of the aqueous extract

The aqueous plant extract of *S. alba* was prepared using the traditional method (Ghazali *et al.*, 2022) by washing the plant parts with D.W. well to remove contaminants from the surface and drying them well with dry air for three days. 50 g of willow bark was ground well and placed in a glass beaker with a capacity of 500 mL that contains 300 mL of D.W. The mixture was heated at 60°C for 30 minutes. Then the extract was filtered using Whatman No. 1 filter paper and stored at 4°C for later use.

2.3 Reagents and chemicals used

All chemicals used in this study were of analytical grade. Silver nitrate (AgNO₃, Sigma-Aldrich, Cat. No. S7653), potassium chloride (KCl, Sigma-Aldrich, Cat. No. P9541), and phosphate-buffered saline (PBS, Gibco, Cat. No. 10010023) were employed during nanoparticle synthesis and experimental procedures. Giemsa stain used for cytogenetic evaluation was obtained from Merck (Cat. No. 1.09204). All reagents were prepared according to the manufacturers' instructions, and the amounts used in each experiment are detailed within the methodological subsections.

2.4 Biosynthesis of silver nanoparticles

Silver nanoparticles (AgNPs) were synthesized according to Subhani *et al.*, (2024) using bark of *S. alba* aqueous extract as a plant-based reducing agent and silver nitrate (AgNO₃) as the silver source. The reaction mixtures were prepared by combining 9 mL of AgNO₃ at varying concentrations (1.0, 1.5, 2.0, and 2.5 mM) with 1 mL of the previously prepared willow bark extract. It should be noted that concentrations expressed in this study (mM) refer to molarity of AgNO₃ used during nanoparticles synthesis while all biological assays were performed using AgNPs synthesized from these precursor molarities. The mixtures were then incubated in a dark chamber at 30°C for 24 hours without agitation to prevent photoreduction of silver nitrate. The selected conditions were adopted from previously reported studies demonstrating efficient green synthesis of stable silver nanoparticles. The selected AgNO₃ concentrations were chosen based on preliminary optimization experiments and in accordance with commonly reported ranges (0.5-5 mM) to ensure controlled nanoparticles formation and stability. These concentrations were specifically selected as they are known to influence nanoparticles formation, including particle size, morphology and yield. The nanoparticles were purified by centrifugation at 10000 rpm for 15 minutes, pellet was washed 3 times with distilled water. Each experiment was performed in triplicate (n = 3) to ensure reproducibility. pH was maintained at 7.0, and visual color change was monitored at regular intervals (every 2 hours) until stabilization. The molarities (1-2.5 mM) refer to the concentration of AgNO₃ used during nanoparticles synthesis. The synthesized AgNPs were used in antimicrobial assays.

All experiments were performed under identical conditions to ensure batch-to-batch reproducibility and each synthesis was conducted in triplicate (n = 3).

2.5 Characterization of the plant-based green synthesized silver nanoparticles

The subsequent methods were used to characterize the plant-based green synthesized nanoparticles. Every test administered in the laboratory of Ministry of Science and Technology, Baghdad, Iraq.

2.5.1 Visual observation

Silver nanoparticles are characterized by a noticeable color change by different period, which serves as an important indicator for the early detection of green-synthesized NPs.

2.5.2 Ultraviolet visible is spectroscopy

One practical approach for identifying the formation of green-synthesized nanoparticles by (Masood *et al.*, 2025) through UV-Vis spectrophotometry. The absorbance spectra were recorded using a UV-Vis spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, USA) over a wavelength range of 200-800 nm.

2.5.3 Atomic force microscopy (AFM) and scanning electron microscopy (SEM)

Atomic force microscopy (AFM) and scanning electron microscopy (SEM) analyses were conducted using an NT-MDT scanning probe microscope. The nanoparticle samples were first diluted with distilled water, then a drop of the diluted solution was placed on a clean glass slide (1×1 cm). After allowing the sample to air-dry completely, the slide was mounted on the AFM sample stage, and imaging was performed following standard operating procedures (Tran *et al.*, 2025).

2.5.4 Antimicrobial activity

Agar well diffusion method was used to evaluate the antimicrobial activity of the synthesized silver nanoparticles. The bacterial sample that used in this study was provided by the Molecular Biology Laboratory for postgraduate research/ Department of Biology/College of Science/University of Baghdad.

2.5.5 Antimicrobial activity of silver nanoparticles

Mueller Hinton agar was prepared by dissolving 38 g of the medium in 1 L of distilled water. The solution was heated until completely dissolved and sterilized by autoclaving at 121°C for 15 min. After cooling to 45–50 °C, the medium was poured into sterile Petri dishes (20–25 mm in depth) under aseptic conditions and allowed to solidify. For antimicrobial testing, two bacterial strains (*Staphylococcus aureus* and *Escherichia coli*) and one fungal strain (*Candida albicans*) were used. A single colony from each strain was inoculated into brain–heart infusion broth and incubated for 18 h at 37°C. After incubation, 1 mL of each culture was transferred into 5 mL of sterile saline and vortexed to achieve homogeneity. The turbidity of the suspensions was adjusted to match 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). By using sterile swabs, the standardized microbial suspensions were evenly spread across the entire surface of the agar plates to ensure uniform distribution. Then, 100 µL of green-synthesized AgNPs from *S. alba* extract were carefully applied to the surface using a sterile micropipette. Four concentrations of AgNPs (100, 75, 50, and 25 µg/mL) were tested. The inoculated plates were incubated at 37°C for 18 h, after which antimicrobial activity was evaluated based on the diameter of inhibition zones formed around each application site.

2.5.6 Animal acclimatization and experimental design

Adult male Swiss Albino mice (*Mus musculus*), aged between 8–10 weeks and weighing 23–27 g, were obtained from the Biotechnology Research Centre at Al-Nahrain University. The animals were housed under standard laboratory conditions for acclimatization. The animals were randomly assigned into three groups, each consisting of Six mice: (1) Control group: Received normal saline. (2) AgNO₃ NP group: received a single dose of 150 mg/kg. (3) *Salix alba* L extract group: a single dose of 400 mg/kg. Following treatment, all animals were sacrificed and femoral bone marrow was extracted to calculate the mitotic index.

2.5.7 Euthanasia and anaesthesia procedures

All procedures involving animals, including anesthesia and euthanasia, were performed in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020). Prior to euthanasia, mice were briefly anesthetized using isoflurane inhalation (2–3% in oxygen) to minimize distress. Euthanasia was performed by cervical dislocation while under anesthesia, as recommended for small laboratory rodents. The dose of colchicine (0.25 mL of 1 mg/mL, intraperitoneal) used for metaphase arrest was applied following standard cytogenetic protocols and did not cause pain or distress at the time of euthanasia.

2.5.8 Chromosome preparation of the mouse bone marrow

The experiment was done according to (Allen *et al.*, 1977) as follow: For cytogenetic preparation, each animal was intraperitoneally injected with 0.25 ml of colchicine (1 mg/ml) to arrest cells in metaphase. Following euthanasia by cervical dislocation, the femur was aseptically extracted, and the bone marrow was flushed using 5 ml of phosphate-buffered saline (PBS, pH 7.2) into a sterile test tube. The suspension was centrifuged at 2000 rpm for 10 min., and the supernatant was discarded. Cells were then treated with 5 ml of 0.075 M potassium chloride (KCl) as a hypotonic solution and incubated at 37°C with intermittent shaking. After a second centrifugation at the same speed and duration, a chilled fixative was added dropwise with gentle agitation to a final volume of 5 ml. The mixture was kept at 4°C for 30 min. and centrifuged multiple times to ensure proper fixation, with the final suspension adjusted to 2 ml of fixative. A few drops (4–5) were dropped vertically from a height of approximately 90 cm onto pre-chilled glass slides to ensure proper

chromosome spreading. After air drying, the slides were stained with Giemsa solution for 15 min. and rinsed with distilled water. Three slides were prepared per animal for cytogenetic examination under the microscope. This section must include enough detail on the data sources and processes so that others can reproduce your research. The selected 150 mg/kg body weight chosen based on previously published studies (Kim *et al.*, 2008; Asharani *et al.*, 2009). The mitotic index was determined by counting metaphase –arrested cells following colchicine treatment. A total of 1000 cells were examined using standard formula: $MI\% = (\text{number of metaphase cells} / \text{total number of counted cells}) * 100$.

2.6 Ethics approval

All animal procedures in this study were reviewed and approved by the Animal Ethics Committee of Al-Nahrain University, College of Biotechnology. The study was conducted in full compliance with institutional guidelines for laboratory animal care and the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020). No additional permits or animal licenses were required for this study; if this information is not available, this is because the institution does not issue separate license numbers for routine academic research involving mice.

2.7 Statistical analysis

The results were statistically analysed using one-way ANOVA followed by Tukey's post-hoc test to compare differences between groups. A significance level of $p < 0.05$ was considered statistically significant. All analyses were carried out using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Data were tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test prior to performing one-way ANOVA followed by Tukey's post hoc test.

3. Results and discussion

3.1 Biogenic silver nanoparticles

The formation of green silver nanoparticles was confirmed through noticeable changes in the color of the reaction mixture as well as spectrophotometric analysis (Hussain *et al.*, 2025). To better illustrate the nanoparticle formation process, image of the reaction mixture at time zero (immediately after adding the extract to AgNO_3) and after 2h of incubation have been included in (Figure 1). The solution initially appeared pale yellow and gradually intensified to light brown, eventually reaching a dark brown color after 24 hours. The darker color observed at 2 h compared to 24 h may be due to aggregation or change in nanoparticles size and distribution over time. This shift in color indicates the successful reduction of silver ions (Ag^+) into elemental silver nanoparticles (Ag^0), likely driven by the active phytochemicals present in the willow bark extract. The brown coloration is commonly linked to the excitation of surface plasmon resonance (SPR), which is characteristic of silver nanoparticles (Muhammad *et al.*, 2025). Spectral analysis showed a distinct absorbance peak around 433 nm, confirming the presence of AgNPs. The intensity of this SPR peak increased with higher concentrations of AgNO_3 , reaching its maximum at 2 mM, suggesting more efficient nanoparticle formation at this concentration. The synthesis process may have been influenced by hydrophilic and hydrophobic interactions among the components, which can promote nanoparticle stability and shape (Hindryawati *et al.*, 2025).

These findings are in line with previous reports showing that silver nanoparticles in aqueous solution typically exhibit brown coloration due to SPR excitation (Khalid *et al.*, 2025). Additionally, factors such as the composition of the biological extract and the concentration of metal salts play a key role in determining nanoparticle yield and characteristics. The unique optical properties of noble metals like silver stem from their ability to support surface SPR, making them ideal for nanoparticle-based applications (Saini *et al.*, 2025) (Figure 2).

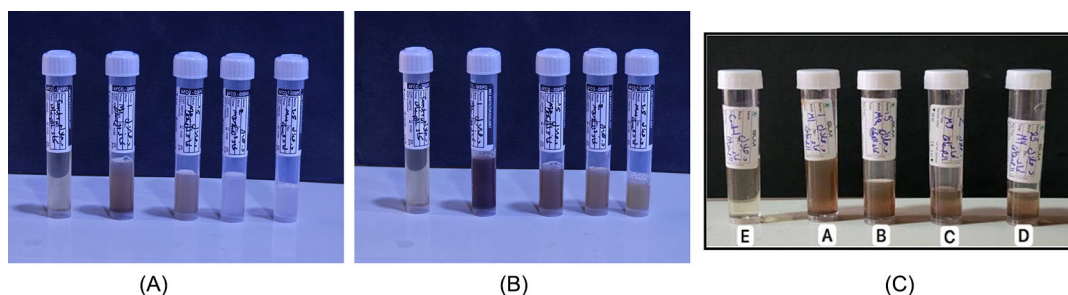


Figure 1. Visual observation of green-synthesized silver nanoparticles at different time (a) zero time, (b) after 2 hours and (c) after 24 hours. (A–D) represent willow bark extract loaded with silver nanoparticles at concentrations of 1, 1.5, 2, and 2.5 mM AgNO_3 , respectively. (E) shows the aqueous willow bark extract without the addition of AgNO_3 .

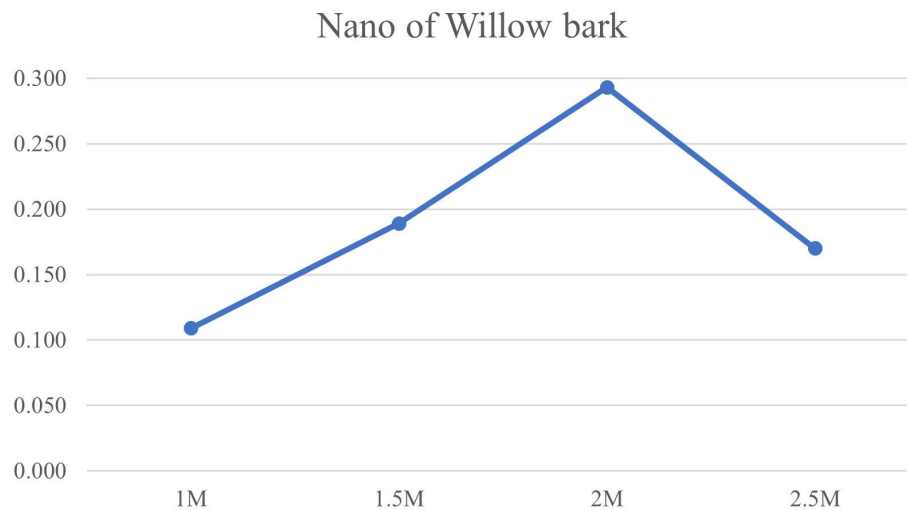


Figure 2. UV-visible absorption spectra of green-synthesized silver nanoparticles, showing a characteristic surface plasmon resonance peak at 433 nm.

Atomic force microscopy (AFM) imaging provided valuable insight into the surface morphology and topography of the green-synthesized silver nanoparticles at varying silver nitrate concentrations. As seen in (Figure 3 A–D), the nanoparticles exhibited distinguishable differences in size distribution and surface roughness depending on the precursor concentration.

At 1 mM (Figure 3-A), the nanoparticles were densely distributed with an average particle size of approximately 79.57 nm and an average height of 75 nm, indicating a relatively uniform structure that similarity to *Jilani et al., (2025)*.

As the concentration increased to 1.5 mM (Figure 3-B), the average size decreased to 69.19 nm with a reduced height of 65 nm. This suggests that a moderate increase in AgNO_3 concentration may enhance the nucleation rate, yielding smaller and more compact nanoparticles due to limited aggregation (*Koçer and Özçimen 2025*).

Interestingly, at 2 mM (Figure 3-C), the average particle size rose to 85.66 nm and the height reached 80 nm. This might be attributed to particle growth dominating over nucleation, likely caused by the saturation of reducing agents in the *S. alba* extract, resulting in fewer but larger particles (*Benaissa et al., 2025*).

However, when the concentration increased to 2.5 mM (Figure 3-D), the particle size again decreased to around 70 nm with a height of 60 nm. This fluctuation indicates that beyond a certain concentration threshold, the stabilizing capacity of the phytochemicals becomes insufficient to control the growth, leading to partial aggregation and size variation (*Elumalai et al., 2025*).

The grain size distribution charts (Figure 4 A-D), further support these observations, showing a shift in peak particle populations depending on the concentration. Most particles fell within the 50–100 nm range, consistent with effective nanoscale synthesis. The narrow size distribution observed, especially at 1.5 and 2.5 mM, suggests enhanced control over particle growth when silver ion concentration and phytochemical reducing agents are well balanced (*Zhu et al., 2025*).

Overall, the AFM results confirm that silver nitrate concentration plays a crucial role in determining nanoparticle size, height, and distribution. Optimal synthesis appears to occur around 1.5 to 2 mM, where particle uniformity and stability are most favorable. These findings highlight the sensitivity of green synthesis to reactant concentrations and the importance of fine-tuning reaction parameters to achieve desirable nanoparticle characteristics (*Wilson et al., 2025*).

As the previous figure which presents AFM-based measurements of the surface heights of green-synthesized silver nanoparticles prepared at varying silver nitrate concentrations. The recorded average heights were 75 nm (1 mM), 65 nm (1.5 mM), 80 nm (2 mM), and 60 nm (2.5 mM), indicating noticeable morphological variation. These differences suggest that the concentration of silver precursor plays a key role in controlling nanoparticle growth and surface topology.

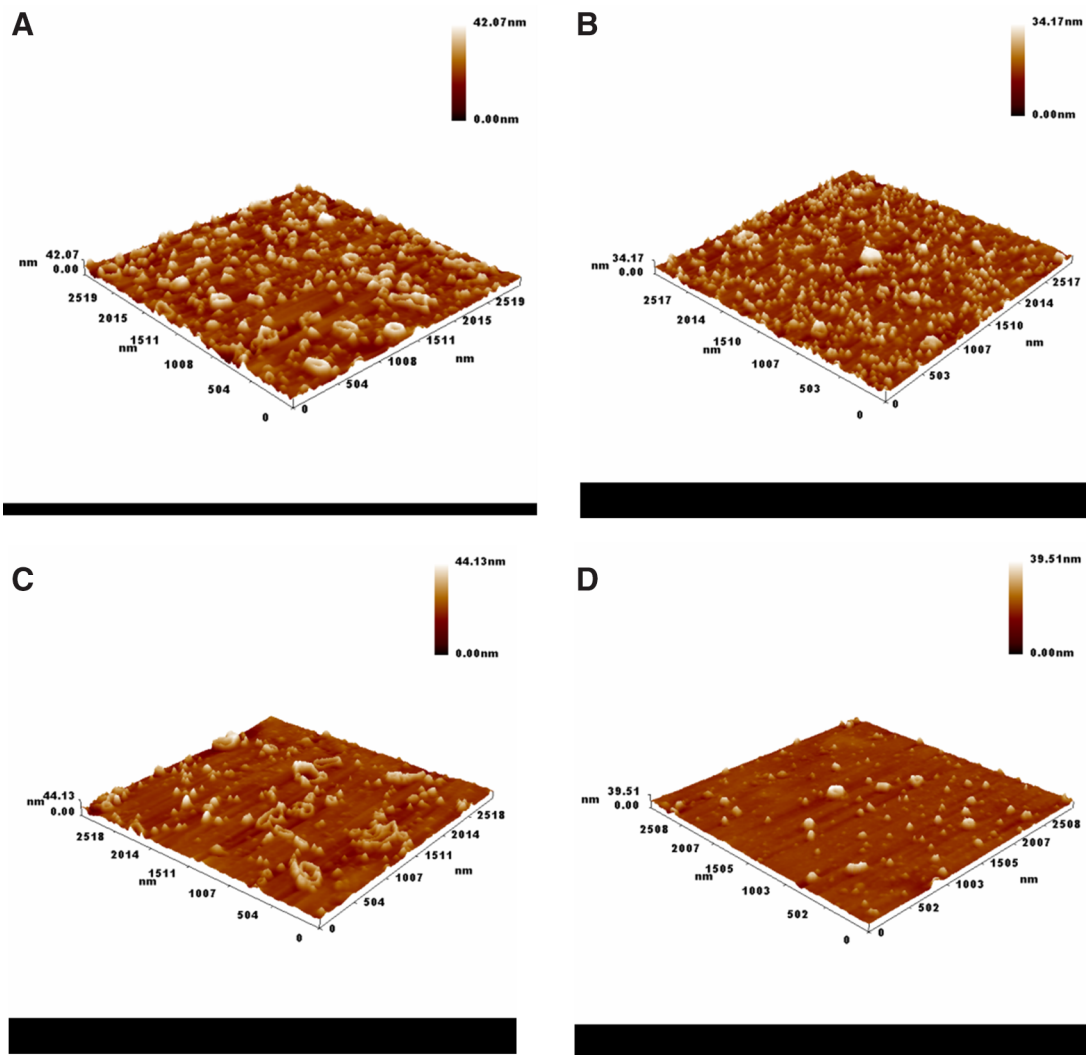


Figure 3. Atomic Force Microscopy (AFM) 3D surface morphology of green-synthesized silver nanoparticles at different AgNO_3 concentrations: (A) 1 mM, (B) 1.5 mM, (C) 2 mM, and (D) 2.5 mM.

The SEM images as [Figure 5 A–D](#) reveal clear differences in surface morphology and particle aggregation of green-synthesized silver nanoparticles at various magnifications. Spherical to semi-spherical particles are visible with varying levels of distribution, indicating successful formation and surface interaction influenced by silver nitrate concentration ([Ahmed and Rahmah, 2025](#)). The SEM images show micrometer-scale structures, which are attributed to nanoparticle aggregation during sample preparation and drying. The features represent agglomerated clusters rather than individual nanoparticles. In contrast, AFM analysis and the size distribution results confirm that the nanoparticles are within the nanoscale range, indicating that SEM observation reflect aggregation rather than actual particle size. The aggregation behaviour is commonly observed in green-synthesized nanoparticles due to the presence of plant-derived biomolecules. It may influence biological activity by reducing surface area and cellular interaction while enhancing stability.

3.2 Antimicrobial activity

The experimental part of the study included several biological assays to evaluate the antimicrobial activity of the prepared extract against selected bacterial (*E. coli* and *S. aureus*) and fungal strains (*C. albicans*) under laboratory conditions, as shown in [Figure 6 A–C](#).

In this study, the aqueous extract of *S. alba* was used to synthesize silver nanoparticles and tested for antimicrobial activity against *E. coli* *in vitro* ([Figure 6-A](#)). Petri dishes containing nutrient medium were treated with different precursor molarity of (AgNO_3) (1.0, 1.5, 2.0, and 2.5 mM). The inhibition zones measured 8, 10, 14, and 15 mm, respectively.

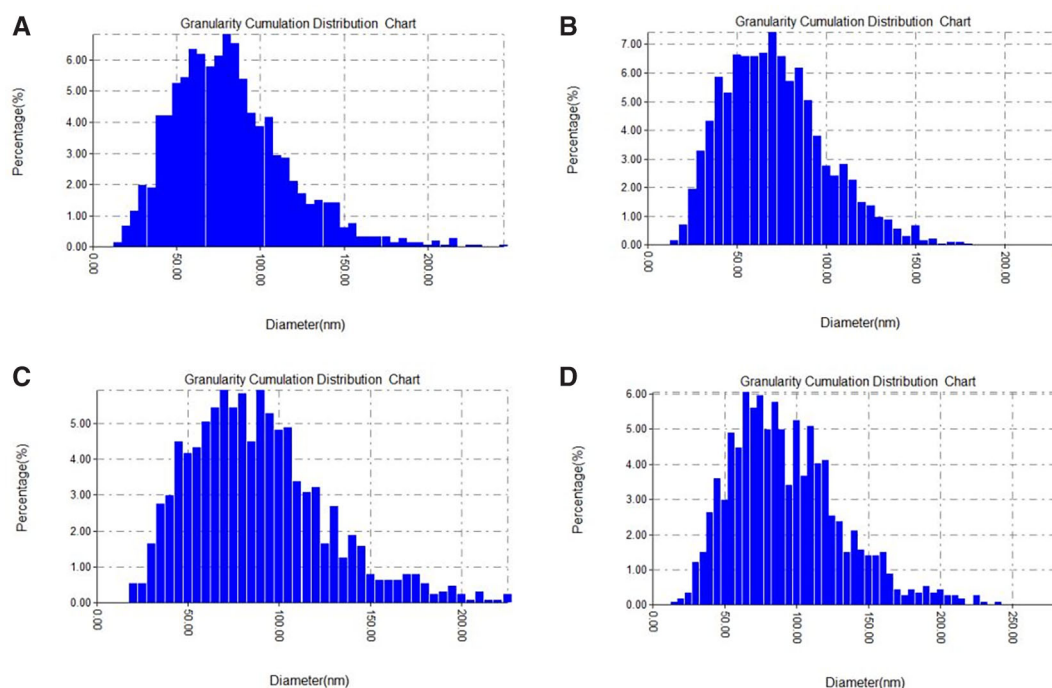


Figure 4. AFM particle size distribution analysis of green-synthesized silver nanoparticles at different AgNO_3 concentrations: (A) average height of 75 nm and 79.57 nm for average size, (B) average height of 65 nm and 69.16 nm for average size, (C) average height of 80 nm and 85.60 nm for average size, and (D) average height of 60 nm and 70 nm for average size.

The gradual increase in inhibition zone diameter with higher concentrations confirms a dependent on the molarity of AgNO_3 used during synthesis antibacterial effect of the synthesized nanoparticles (Agrawal *et al.*, 2025).

Where, the antibacterial activity of the *S. alba* based silver nanoparticle extract was evaluated against *S. aureus* using a nutrient-rich medium inoculated with the bacterial strain. Different precursor molarity of AgNO_3 (1, 1.5, 2, and 2.5 mM) produced inhibition zones measuring 13, 15, 17, and 18 mm, respectively. Previous studies which Turay (2025) who estimated that the gradual increase in zone diameter with higher concentrations confirms a positive correlation between extract dose and antibacterial effectiveness. Although, Shalaby *et al.*, (2025). The gradual increase in zone diameter with higher concentrations confirms a positive correlation between extract dose and antibacterial effectiveness. This enhanced activity at elevated concentrations may be attributed to the increased availability of silver nanoparticles and phytochemicals, which together intensify cell wall disruption, protein denaturation, and oxidative stress within the bacterial cells (Wang *et al.*, 2025), as shown in Figure 6-B.

Furthermore, the antifungal activity of the green-synthesized AgNO_3 NPs was evaluated against *C. albicans*. As shown in the Figure 6-C, four different concentrations of the *S. alba* NPs extract that have different molarity concentration (1, 1.5, 2, and 2.5 mM) were tested. Clear inhibition zones were observed around each well (13, 15, 17 and 18 mm), respectively, with their diameters increasing proportionally with concentration (Ibrahim *et al.*, 2025). This suggests a dependent on the molarity of AgNO_3 used during synthesis antifungal effect, likely due to enhanced NPs interaction with the fungal cell membrane. The increased presence of bioactive compounds and silver ions may contribute to membrane disruption, oxidative stress, and enzyme inhibition in *C. albicans* (Beyatli, 2025).

When compared with other plant-mediated silver nanoparticles, the antimicrobial performance of *S. alba* appears to be equally strong or superior. For example, *Cassia siamea*-derived AgNPs produced inhibition zones of 10–14 mm against *S. aureus* (Turay, 2025), while *Acacia mangium*-based AgNPs showed zones of 11–15 mm against *E. coli* (Aji *et al.*, 2025). Similarly, *Xanthium strumarium*-mediated nanoparticles demonstrated inhibition zones around 12–15 mm against *C. albicans* (Beyatli, 2025). In our study, *S. alba*-derived AgNPs exhibited larger inhibition zones (up to 18 mm against *S. aureus* and *C. albicans*), suggesting that the phytochemical composition of willow bark rich in salicin, flavonoids, and phenolic compounds may enhance both nanoparticle stability and biological activity. These comparative results highlight the distinctive contribution of *S. alba* among plant-based nanoparticle systems.

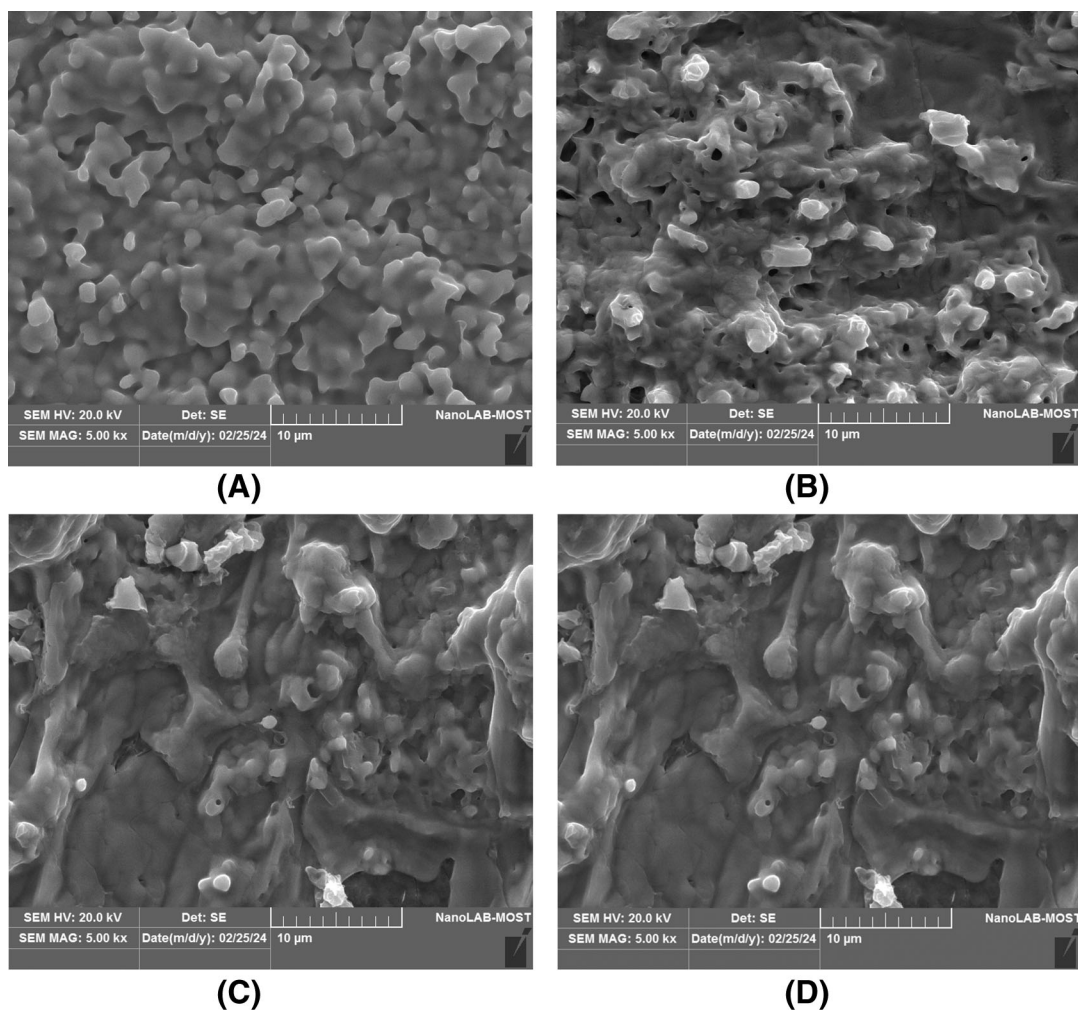


Figure 5. Scanning electron microscopy (SEM) images of green-synthesized silver nanoparticles at different silver nitrate concentrations: (A) 1 mM, (B) 1.5 mM, (C) 2 mM, and (D) 2.5 mM. All images were captured at a magnification of 5.00 kx with a uniform scale bar of 10 μm.

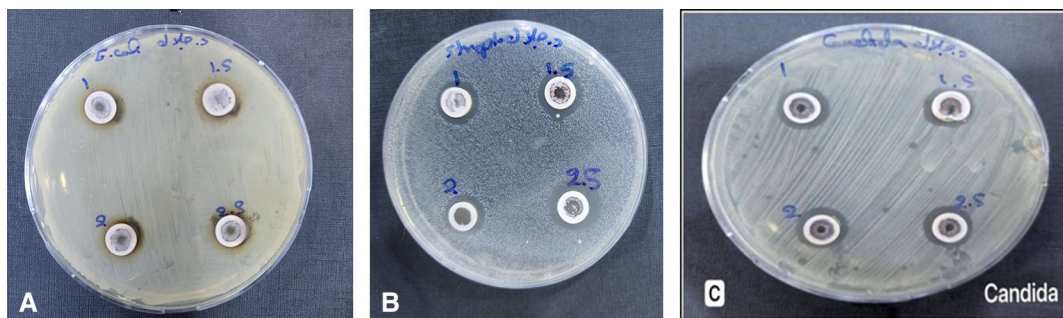


Figure 6. Antibacterial activity of silver nanoparticles synthesized using aqueous extract of *Salix alba* L using AgNPs synthesized from different precursor of AgNO_3 (1, 1.5, 2, and 2.5 mM) against (A) *Escherichia coli*, (B) *Staphylococcus aureus*, and (C) *Candida albicans*.

The antimicrobial and cytogenetic effects of AgNPs generally belong to a combination of physical, chemical, and biochemical mechanisms rather than a single pathway. At the microbial level, AgNPs can adhere to the negatively charged bacterial cell wall, damaging membrane integrity and increasing permeability, which cause leakage of essential ions and metabolites (Ibrahim *et al.*, 2025). Once internalized, AgNPs release silver ions (Ag^+), which interact with sulfur- and phosphorus-containing biomolecules such as proteins and DNA, thereby impairing enzymatic activity and replication processes (Beyatli, 2025). Another well-documented mechanism is the generation of reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals, and hydrogen peroxide, which induce oxidative stress, damage lipids, oxidize proteins, and fragment nucleic acids (Wang *et al.*, 2025). These results are supported by previous studies (Darwich *et al.*, 2025; Aljohani *et al.*, 2026) which indicated that biosynthesized silver nanoparticles have antibacterial activity because of different mechanisms like, damage of cell membrane, induction of oxidative stress and bacterial DNA and proteins interaction.

3.3 Chromosome from somatic cells of the mouse bone marrow

The results clearly indicated a decrease in mitotic index in silver nanoparticles treated group as compared with negative control. While it increased in plant extract group to 7.8% as compared to negative group 5%. As shown in Table 1 and Figure 7 (A-B).

At the cytogenetic level, AgNPs may directly interact with chromosomes and mitotic machinery. Several studies have demonstrated that AgNPs can cause chromosomal aberrations, spindle disruption and disruption of cell-cycle progression, which is consistent with the observed reduction in mitotic index (Tang, 2025; Osman *et al.*, 2025). For cancer therapy for the recent decades natural products play an important role in the development of a drug. In addition to the

Table 1. Mitotic index (%) values of bone marrow cells in mice treated with AgNP and plant extract compared with the control group.

Groups	Mitotic index % (M $\pm$ SD)	P value
Negative control	5.0 $\pm$ 0.67 ^a	p < 0.001 $\rightarrow$ ***
AgNP-treated AgNPs (150 mg/kg)	1.2 $\pm$ 0.31 ^b	p < 0.001 $\rightarrow$ ***
Plant extract treated (400 mg/kg of <i>Salix alba</i> L)	7.8 $\pm$ 0.31 ^c	p < 0.001 $\rightarrow$ ***

***Indicates a highly statistically significant difference (p < 0.001).

Different superscript letters (a, b, c) indicate statistically significant differences between groups according to the post hoc multiple comparison test, where groups with different letters differ significantly at p < 0.05.

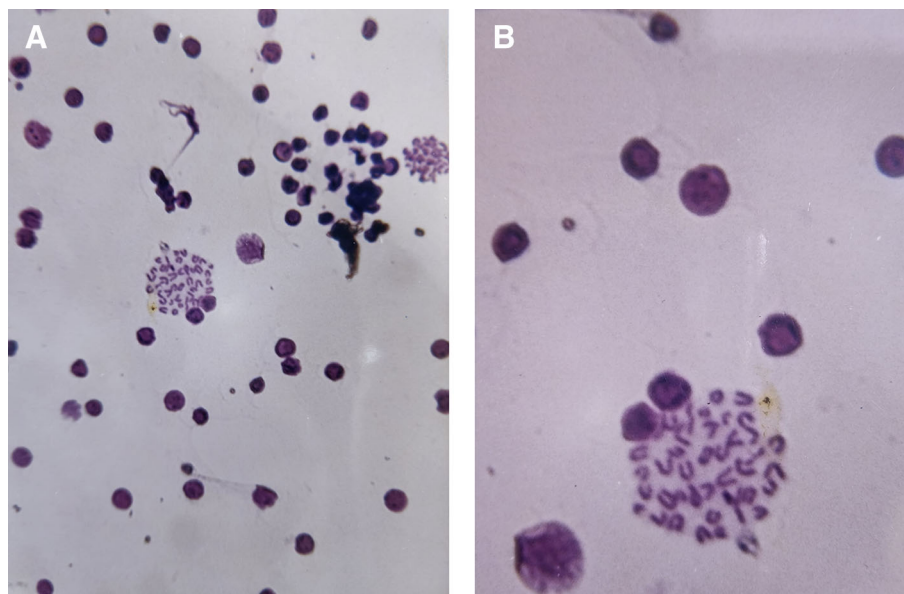


Figure 7. Cytogenetic effects of aqueous extract from *Salix alba* L in mice bone marrow cells. (A) Representative microscopic image showing a general field view of bone marrow cells. (B) Magnified view highlighting mitotic cells (400X).

possible relationship between natural products and regulation of telomerase working (Shalash *et al.*, 2021). ROS-mediated DNA strand breaks, coupled with impaired repair pathways, further contribute to chromosomal instability (Zhang *et al.*, 2025). Additionally, AgNPs have been reported to interfere with mitochondrial respiration, leading to ATP depletion and triggering apoptosis, which indirectly reduces the pool of actively dividing cells (Bentrad, 2025).

It is also worth noting that the biological response to AgNPs is influenced by multiple factors, including nanoparticle size, shape, surface charge, and the phytochemical composition of the reducing agent used in synthesis (Kirubakaran *et al.*, 2025). The phytochemicals in *S. alba* extract such as flavonoids, tannins, and salicin are likely to play an important role: (i) reducing and stabilizing silver nanoparticles, and (ii) exerting protective antioxidant effects that mitigate genotoxicity. This may explain why the group treated with *S. alba* extract alone showed normal or elevated mitotic index, in contrast to the significant suppression observed in the AgNP-treated groups. Such duality highlights the importance of considering both nanoparticle characteristics and plant-derived biomolecules when evaluating biosafety as explained in (Chaudhary *et al.*, 2025b).

Mitotic index in AgNP-treated groups decreased which can be mechanistically explained by nanoparticle-induced oxidative stress and genotoxic effects. Silver nanoparticles (AgNPs) have the ability to penetrate cellular membranes and generate reactive oxygen species (ROS), which cause damage of DNA, proteins, and lipids, led to disruption of cell-cycle progression and inhibition of mitosis. Several recent studies have demonstrated that AgNP exposure induces DNA strand breaks, chromosomal abnormalities, and cell-cycle arrest, which collectively affect cellular proliferation (Huang *et al.*, 2025; Sánchez-Alarcón *et al.*, 2025). A significant decrease in mitotic index have observed in vivo studies on bone marrow cells following AgNP exposure due to genotoxic stress and chromosomal damage (Mahmood & Ahmed, 2024).

In contrast, the relatively higher mitotic index observed in plant extract-treated groups may be attributed to the antioxidant activity of phytochemicals present in *Salix alba* L. This plant is rich in phenolic compounds, flavonoids, and related bioactive molecules that possess strong free radical scavenging properties. These compounds can reduce oxidative stress by neutralizing ROS and protecting cellular components from nanoparticle-induced damage, thereby preserving normal cell division. Recent studies have highlighted the role of plant-derived polyphenols in mitigating oxidative damage, enhancing cellular defense mechanisms, and supporting cell survival and proliferation under stress conditions (Sharma *et al.*, 2024; Muscolo *et al.*, 2024).

The cytogenetic effects by MI which reported in the present study are comparable to those reported for other plant mediated AgNPs in recent literature. In this study, AgNP-treated groups showed a marked reduction in MI ($1.2 \pm 0.14\%$) as compared to negative control ($5 \pm 0.30\%$). Similar reduction in previous studies, where plant mediated AgNPs decreased MI to 1-3% in bone marrow cells, depending on nanoparticles size, dose, and synthesis method (Mahmood & Ahmed, 2024; El-Naggar *et al.*, 2022).

Overall, the results demonstrate that *S. alba* mediated AgNPs exhibit strong antimicrobial and measurable cytogenetic effects in a dose-dependent manner. To better interpret these outcomes, that compare our findings with previous studies and discuss possible mechanisms that may explain the observed biological activities.

4. Conclusion

This study demonstrates the successful green synthesis of AgNPs using aqueous *S. alba* bark extract, confirmed by visual color change, UV-Vis spectroscopy, AFM, and SEM analyses. The biosynthesized AgNPs exhibited significant antimicrobial activity against *E. coli*, *S. aureus*, and *C. albicans*, with inhibition zones increasing dependent on precursor molarity of AgNO₃. Additionally, cytogenetic evaluation indicated that while chemically synthesized AgNPs reduced the mitotic index, the *S. alba* extract maintained or enhanced mitotic activity, suggesting a protective biological effect. These findings highlight the potential of *S. alba* based AgNPs as antimicrobial agents. However, their biocompatibility should be interpreted with caution and within the limits of the present study. Further investigations required to fully evaluate their safety and suitability for biomedical applications.

Data availability

The raw data supporting the findings of this study are publicly available in the Figshare repository at <https://doi.org/10.6084/m9.figshare.30944873> (AlMaliky, 2025a). The dataset includes raw atomic force microscopy (AFM) images and corresponding instrument output reports, raw antimicrobial inhibition zone measurements against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, and raw cytogenetic mitotic index data. The data are openly accessible with no embargo or login requirement and are available under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) license. The completed ARRIVE 2.0 checklist for reporting animal research is also publicly available on Figshare at <https://doi.org/10.6084/m9.figshare.31046287> (AlMaliky, 2025b).

Extended data

Extended data supporting this study are available in a public repository. The extended data includes raw atomic force microscopy (AFM) images and instrument output reports, raw antimicrobial inhibition zone measurements against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, and raw cytogenetic mitotic index data.

Acknowledgements

The author expresses their appreciation to the Biotechnology department at the University of Al-Nahrain, Iraq, for their valuable assistance.

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Applied Genetics, Karnatak University Dharwad, Dharwad, Karnataka, India

Akshata Choudhari Padti

Applied Genetics, Karnatak University Dharwad, Dharwad, Karnataka, India

The authors have comprehensively addressed all comments and suggestions raised during the review process. The manuscript has been substantially improved, and the revised version is now suitable for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Green synthesis of nanoparticles, nanoparticle characterization, and biological applications.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 05 June 2026

<https://doi.org/10.5256/f1000research.198859.r490719>

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Nourhane A. Darwich 

Biological Science, Beirut Arab University, Beirut, Lebanon

The authors have adequately addressed all reviewer comments and implemented the required revisions. The manuscript has been significantly improved in terms of clarity, presentation, and

scientific quality. Therefore, I recommend the paper for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Green synthesis of nanoparticles; nanobiotechnology; in vitro and in vivo experimental models; nanotoxicology; oxidative stress and antioxidant assays; antimicrobial activity; antidiabetic studies; nanoparticle characterization (UV-Vis, FTIR, XRD, TEM, SEM, XPS, DLS); histopathology and biological evaluation of nanomaterials; molecular docking and computational analysis.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 01 April 2026

<https://doi.org/10.5256/f1000research.190246.r470711>

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Nourhane A. Darwich

Biological Science, Beirut Arab University, Beirut, Lebanon

This manuscript presents an interesting study on the green synthesis of silver nanoparticles using *Salix alba* extract and evaluates their antimicrobial and cytogenetic effects. The topic is relevant and aligns with current trends in sustainable nanotechnology. However, several methodological and interpretative aspects require clarification to improve the scientific rigor and reproducibility of the study.

Question to author

1. Could the authors clarify the rationale behind selecting the specific AgNO₃ concentrations (1–2.5 mM)?
2. Were optimization studies (pH, temperature, reaction time) performed before selecting the final synthesis conditions?
3. How was batch-to-batch reproducibility of nanoparticle synthesis ensured? \
4. The study relies mainly on UV-Vis, AFM, and SEM. Why were DLS, zeta potential, or XRD not included to confirm size distribution, stability, and crystallinity?
5. The UV-Vis peak at ~433 nm confirms nanoparticle formation, but can the authors provide full spectra and peak width analysis for better interpretation?
6. SEM images (Figure 5) report particle sizes in micrometers, which contradict nanoscale findings from AFM. Could the authors clarify this discrepancy?

7. Is there evidence of nanoparticle aggregation in SEM images, and how might this affect biological activity?
8. Why was only the agar well diffusion method used instead of more quantitative methods such as MIC or MBC?
9. Were positive controls (e.g., standard antibiotics) included for comparison?
10. How was the concentration of AgNPs (mg/mL) calculated, given that synthesis was based on molarity (mM)?
11. The study includes only three experimental groups. Why was a nanoparticle dose-response design not included for cytogenetic analysis?
12. The dose of AgNPs (150 mg/kg) appears relatively high—what is the justification for this dose selection?
13. Could the authors explain how many cells were counted per slide for mitotic index calculation?
14. The manuscript mentions ANOVA with Tukey's test. Were assumptions (normality, homogeneity of variance) verified before analysis?
15. Why are standard deviations or confidence intervals not consistently reported alongside mean values?
16. Some conclusions suggest "biocompatibility," yet cytogenetic results show reduced mitotic index. Can the authors better reconcile this apparent contradiction?
17. The discussion largely describes results; could the authors provide deeper mechanistic insight supported by recent literature?
18. How do these findings compare quantitatively with other plant-mediated AgNPs reported in recent studies?
19. Several grammatical and typographical errors are present throughout the manuscript. Careful language editing is recommended.
20. Figures require improved clarity, labeling, and consistency (e.g., units, magnification, scale bars).
21. Some sections of the methods lack sufficient detail to ensure reproducibility (e.g., nanoparticle purification steps).
22. I recommend that the authors incorporate additional relevant references to strengthen the discussion, particularly regarding nanoparticle characterization techniques and the antibacterial activity of silver nanoparticles
Refer to reference 1&2

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Green synthesis of nanoparticles; nanobiotechnology; in vitro and in vivo experimental models; nanotoxicology; oxidative stress and antioxidant assays; antimicrobial activity; antidiabetic studies; nanoparticle characterization (UV-Vis, FTIR, XRD, TEM, SEM, XPS, DLS); histopathology and biological evaluation of nanomaterials; molecular docking and computational analysis.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 30 March 2026

<https://doi.org/10.5256/f1000research.190246.r470719>

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Santosh Mallikarjun Bhavi 

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The manuscript presents an interesting study on the green synthesis of silver nanoparticles using *Salix alba* bark extract and their antimicrobial and cytogenetic effects. The topic is relevant and has potential applications in nanobiotechnology. However, several issues related to methodological clarity, citation accuracy, data presentation, and overall consistency need to be addressed before the manuscript can be considered for indexing. The following comments are provided to help improve the quality and clarity of the work.

1. Authors are requested to mention the complete scientific name of the plant at first mention; is it *Salix alba* L.? Please confirm and maintain consistency throughout the manuscript.
2. In Section 2.1, authors mentioned collection of leaves, whereas throughout the study bark

- extract is used. Authors need to clarify whether leaves or bark were actually used, as this creates confusion and affects reproducibility.
3. In the Introduction, the sentence “Traditionally recognized for its therapeutic potential...”—the citation needs to be changed or the sentence needs to be revised, as the cited paper discusses the *Salix* genus, whereas the sentence specifically refers to *S. alba*. Kindly update it accordingly.
 4. In the Introduction, the statement regarding synthesis of silver nanoparticles cites a paper that appears to be about gold nanoparticles. Authors are requested to cross-check this and all other citations carefully for similar issues.
 5. Is the bark sample heated during preparation of the aqueous extract? If yes, mention the temperature and time in Section 2.2.
 6. In Section 2.4, the phrase “willow bark of *S. alba*” is slightly redundant and can be improved for clarity and scientific precision. Consider revising it to “*Salix alba* bark” or “bark of *Salix alba*”.
 7. In Section 2.5.2, do not include discussion or expected results such as the peak at 433 nm in the Methods section. This should be moved to the Results and Discussion section. Also, include the scanning range of the UV-Vis spectrophotometer and the instrument details.
 8. If possible, authors are encouraged to perform XRD for crystalline nature, FTIR for functional group analysis, and EDX for elemental composition for better characterization.
 9. Authors should clearly mention which method was used for antimicrobial activity (agar well diffusion or disc diffusion). Why was a standard antibiotic not used as a control in the antimicrobial study? This is necessary. Also, the concentration of AgNPs used (25–100 mg/mL) seems too high. Please check whether this is a typographical error (μg instead of mg). Furthermore, the study is mentioned as dose-dependent, but Figure 6 shows different molarity of synthesized AgNPs rather than different concentrations of AgNPs. Authors need to clarify this.
 10. There is also a conceptual confusion between AgNO_3 molarity used during synthesis and AgNP concentration used in biological assays. Authors should clearly distinguish between these.
 11. In Figure 1, the authors have shown one image of AgNO_3 and another image after mixing AgNO_3 with bark extract (for different molarities). However, it would be clearer if the authors include an image of the reaction mixture immediately after adding the bark extract to AgNO_3 (time zero) and another image of the same mixture after a certain incubation time, showing the color change due to bioreduction. This would better demonstrate the nanoparticle formation process.
 12. In Figure 2, authors should plot the complete UV-Vis spectrum for all four molarity-based synthesized AgNPs, not only at 433 nm. Please use standard software such as Origin. Also, change the title from “Nano of the willow bark” to “UV-visible spectroscopy”.
 13. Why did authors use different scale bars for SEM images (e.g., 5 μm , 50 μm , 2 μm , and 10 μm)? This creates confusion and makes comparison difficult. Since four different types of AgNPs were synthesized (based on different AgNO_3 molarities), authors should present SEM images of all four samples using a common scale bar for proper comparison in the main manuscript. If needed, images with different magnifications can be included in the supplementary data.
 14. For chromosomal study, authors mentioned “single dose of AgNO_3 NPs (150mg/kg)”. Is this referring to AgNPs? If yes, which molarity-based synthesized sample was used? Also, Figure 7 shows only extract-treated cells; what about AgNP-treated cells?
 15. Authors should clearly describe how mitotic index was calculated, including the number of

cells counted and the criteria used.

16. The rationale for selecting the dose (150 mg/kg) is not mentioned; authors should justify this.
17. Some conclusions, such as claims about safety or biocompatibility, seem overstated based on the presented data and should be moderated.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Green synthesis of nanoparticles, nanoparticle characterization, and biological applications.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Apr 2026

Worood Al Maliky

we sincerely thank the reviewer for the positive evaluation and constructive comments. All suggestions have carefully considered and addressed in the revised manuscript.

1. Thank you for your valuable comment. The complete scientific name of the plant is *Salix alba* L. This has been verified and is now correctly stated at the first mention, with consistent usage maintained throughout the manuscript

2. We would like to clarify that the reference to "leaves" in Section 2.1 was a typographical error. The study was performed using the bark extract of *Salix alba* L.. This has been

carefully revised and corrected throughout the manuscript to avoid any confusion and to ensure reproducibility.

3.The sentence has been revised to correctly reflect that the cited reference refers to the *Salix* genus rather than specifically to *Salix alba* L.. The text has been updated accordingly to ensure accuracy and consistency.

4.We have carefully reviewed the cited references and confirmed that references [4] and [5] are directly relevant to the synthesis of silver nanoparticles. Reference [3], which discusses nanoparticles in general, has been retained only where appropriate for general statements. All citations have been revised to ensure accuracy and proper alignment with the text.

5.The extraction procedure has been clarified in Section 2.2, including the temperature (60 °C) and extraction duration (30 minutes), to ensure reproducibility of the method

6.The phrase has been revised to “*Salix alba* bark” to improve clarity.

7.The description of the UV-Vis analysis has been revised by removing result-related statements and including the instrument model (SmartSpec™ 3000, Bio-Rad Laboratories, USA) and scanning range (200–800 nm) in Section 2.5.2.

8.Thank you for your insightful suggestion. We agree that XRD, FTIR, and EDX analyses would provide additional information regarding crystallinity, functional groups, and elemental composition. However, due to limited access to these advanced characterization facilities, these analyses could not be performed in the current study. Nonetheless, nanoparticle formation was adequately confirmed through UV-Vis spectroscopy and supported by AFM and SEM analyses.
Future studies will include these analyses for further validation.

9.The antimicrobial method has been clarified as the agar well diffusion method in Section 2.5.4.

Regarding the absence of a standard antibiotic control, we acknowledge its importance. However, the primary aim of this study was to investigate the relative antimicrobial activity of the synthesized AgNPs. This limitation has now been addressed and discussed in the revised manuscript.

Concerning the reported concentrations (25–100 mg/mL), we appreciate the reviewer’s observation. This was indeed a typographical error, and the correct unit is µg/mL, which has now been corrected throughout the manuscript.

Finally, we agree with the reviewer regarding the terminology used. The antimicrobial activity presented in Figure 6 corresponds to different molarity-based synthesized AgNPs, rather than different applied doses. Therefore, the term “dose-dependent” has been revised to “concentration-dependent” to accurately reflect the experimental design.

10.Thank you for this important and insightful comment. We agree that there was a lack of clarity in distinguishing between the molarity of AgNO₃ used during nanoparticle synthesis and the concentration of AgNPs used in the biological assays.
In the original version, the values expressed in mM were mistakenly described as “extract

concentrations,” which may have caused confusion. These values in fact refer to the AgNO_3 precursor molarity used during the synthesis of AgNPs, not to the concentration of AgNPs in the antimicrobial assay.

The manuscript has now been carefully revised to clearly distinguish between these two parameters. All relevant sections have been corrected by replacing terms such as “extract concentrations” and “concentration-dependent” with more accurate expressions, including “AgNPs synthesized from different AgNO_3 precursor molarities.”

11. In response, we have included additional images to better illustrate the nanoparticle formation process. Specifically, images of the reaction mixture were added at: 0 h (immediately after mixing), and 2 h after incubation,

The observed variation in color intensity over time suggests dynamic changes in nanoparticle formation, aggregation, and stabilization processes. Image have been uploaded as revised figure files and data availability

12. Thank you for this valuable suggestion. We agree that presenting the complete UV-Vis spectra for all synthesized AgNP samples would provide a more comprehensive characterization of the nanoparticles.

However, the UV-Vis analysis was conducted in a collaborating laboratory outside the country, and due to current logistical constraints and limited access to the original instrumental data, it is not possible at this stage to retrieve or reprocess the full spectral datasets.

Nevertheless, the characteristic surface plasmon resonance (SPR) peak at 433 nm has been reported, which is widely recognized as a reliable indicator of AgNP formation. The manuscript has been revised to clarify this point.

In addition, the figure title has been corrected from “Nano of the willow bark” to “UV-visible spectroscopy” as recommended

13. In response, the SEM images of all four AgNP samples (corresponding to different AgNO_3 molarities) have been revised and are now presented using a uniform scale bar to allow proper comparison, as recommended.

The updated SEM images have been uploaded as revised figure files and linked to the corresponding figure (Figure 5) in the manuscript.

Additional images at different magnifications remain available in the Data Availability section

14. The term “ AgNO_3 NPs” has been corrected to “AgNPs” throughout the manuscript. The chromosomal study was conducted using three experimental groups: a negative control group, an AgNP-treated group (150 mg/kg body weight), and an extract-treated group (150 mg/kg body weight). The in vivo study was designed as a dose-based evaluation rather than a comparison between different AgNO_3 molarity-based synthesized samples.

Additionally, Figure 7 has been revised and clarified to indicate the respective treatment groups.

No noticeable morphological differences were observed in mitotic cell appearance among the control, AgNP-treated, and extract-treated groups. The effects of the treatments were

primarily quantitative, as reflected by changes in the mitotic index, rather than qualitative changes in mitotic morphology.

15. The mitotic index (MI) was calculated based on metaphase-arrested cells due to the use of colchicine, which inhibits spindle fiber formation and arrests cells at the metaphase stage.

Therefore, only cells observed in metaphase were considered as dividing cells. A total of 1000 cells were examined under a light microscope (40×), and the MI was calculated using the standard formula:

$$\text{MI (\%)} = (\text{Number of metaphase cells} / \text{Total number of cells counted}) \times 100.$$

This has now been clarified in the revised manuscript.

The mitotic index (MI) was determined by counting metaphase-arrested cells following colchicine treatment. Only cells observed in metaphase were considered as dividing cells. This approach is commonly used in cytogenetic studies to enhance the visibility of chromosomes and improve scoring accuracy.

16. The selected dose of 150 mg/kg body weight was chosen based on previously published studies reporting similar non-lethal and biologically effective ranges for silver nanoparticles (Kim et al., 2008; Asharani et al., 2009). "within similar rang

17. We thank the reviewer for this valuable comment.

We agree that the statements regarding safety and biocompatibility may have been overstated. Accordingly, the conclusions have been revised to present a more cautious interpretation of the results.

The claims have been modified to indicate that the findings suggest potential biocompatibility under the tested conditions, rather than making definitive conclusions about safety.

Competing Interests: The authors declare that they have no competing interests.

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