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Research Article

Green Synthesis of Silver Nanoparticles Using Bixa Orellana Seed Extract: Antioxidant, Anticancer, Antibacterial, and Antiproliferative Evaluation

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ABSTRACT

This study presents a novel green synthesis approach for silver nanoparticles (AgNPs) using aqueous seed extract of Bixa orellana. The phytochemical-rich extract, containing flavonoids and carotenoids, effectively reduced silver ions and stabilized the resulting nanoparticles under ambient conditions. Formation of AgNPs was confirmed by UV-visible spectroscopy through a characteristic surface plasmon resonance (SPR) peak at 420 nm. X-ray diffraction (XRD) analysis revealed diffraction peaks at 2θ values of 38.1° , 44.2° , 64.6° , and 77.5° , corresponding to the (111), (200), (220), and (311) crystalline planes. Fourier transform infrared (FTIR) spectroscopy identified functional groups responsible for reduction, capping, and stabilization. Morphological characterization using field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) confirmed nanoscale dimensions, supported by dynamic light scattering (DLS) measurements. The biosynthesized AgNPs demonstrated significant antioxidant, anticancer, antibacterial, and antiproliferative properties, suggesting potential applications in biomedical fields. This environmentally friendly approach presents a sustainable alternative to conventional synthesis methods, utilizing the natural reducing and stabilizing capabilities of Bixa orellana seed extract.

INTRODUCTION

Since the late 20th century, nanotechnology has emerged as a rapidly advancing and interdisciplinary field that integrates concepts from physics, chemistry, materials science, biochemistry, and medicine. The term

"nanotechnology" encompasses a wide range of techniques and applications involving the design, manipulation, and control of matter at the nanoscale typically at the atomic or molecular level to achieve extremely high precision and ultra-fine dimensions (Salata, 2004).

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In recent years, nanomaterials have attracted significant interest across various scientific domains due to their distinctive physical, chemical, and biological characteristics. These materials are increasingly being applied in diverse fields such as medicine, chemical sensing, drug delivery, cosmetics, catalysis, separation science, and electronics (Gross et al., 2007). Among the various forms of nanomaterials, nanoparticles (NPs)—which are clusters of atoms ranging in size from 1 to 100 nanometers in at least one dimension—play a pivotal role. These particles possess unique physical and chemical properties that differ significantly from their bulk counterparts, including high surface area-to-volume ratios, elevated surface energies, consistent shape and size, and enhanced optical, mechanical, and magnetic properties (Khan et al., 2019). Nanoparticles can be produced through a variety of techniques, including physical, chemical, and biological approaches. However, when prioritizing environmental friendliness, low toxicity, cost-efficiency, and time-effectiveness, physical and chemical approaches are often thought to be less optimal, especially when creating silver nanoparticles. As a result, biological or "green" synthesis methods have gained traction as sustainable alternatives. These include the use of microorganisms, enzymes, and plant extracts (Chung et al., 2016). Plant-based synthesis is particularly appealing due to the non-toxic nature of botanical sources, making them ideal candidates for eco-friendly nanoparticle production. Various parts of plants have been used for this purpose, including flowers, leaves, fruits, fruit peels, bark and seeds (Masudulla et al., 2021). Recent studies have demonstrated the successful synthesis of silver nanoparticles using extracts from plants such as ginger (*Zingiber officinale*), which exhibited significant antimicrobial properties (Chondhe et al., 2024). Despite the abundance of studies in this area, ongoing research continues to focus on plant-mediated nanoparticle synthesis, especially for scaling up to economically feasible commercial production.

Researchers are constantly seeking more efficient, environmentally friendly methods to produce nanoparticles that maintain stability and exhibit enhanced functional properties in their intended applications (Niederberger, 2013). In recent years, the use of natural products—particularly plant-based approaches—for the synthesis of metal nanoparticles has gained significant momentum. This surge in interest stems from the environmentally friendly, biodegradable, and non-toxic nature of such methods (Raveendran et al., 2003). Within plant-mediated nanoparticle synthesis, plant extracts function as dual agents: they supply reducing compounds necessary for converting metal ions (such as silver ions) into metallic nanoparticles, and they also act as capping agents to stabilize the newly formed particles. Phytochemicals such as flavonoids, alkaloids, phenols, tannins, steroids, and saponins found in plant extracts are primarily responsible for facilitating the green synthesis of nanoparticles. These natural compounds not only assist in reducing metal ions but also enhance the stability of the synthesized nanoparticles (Ahmed et al., 2016). Although various metals have been explored for nanoparticle synthesis, silver remains one of the most widely used due to its historical significance and broad utility in medical applications (Strohal et al., 2005). Silver nanoparticles (AgNPs) continue to attract significant research interest due to their excellent chemical stability, catalytic activity, electrical conductivity, antibacterial properties, and localized surface plasmon resonance. These properties make AgNPs especially valuable in biomedical applications such as drug delivery, imaging, wound healing, biolabeling, and dental treatments (Shameli et al., 2012). Phytochemicals derived from plants—such as polysaccharides, phenolic acids, flavonoids, and alkaloids—not only reduce metal ions efficiently but also play a crucial role in stabilizing nanoparticles. These compounds help prevent the aggregation and uncontrolled growth of nanoparticles by ensuring colloidal stability (Xulu et al., 2022).



The primary aim of the present study is to synthesize and characterize silver nanoparticles using an aqueous seed extract of *Bixa orellana*, commonly known as the Lipstick Tree, Annatto, or Achiote. This medium-sized evergreen shrub can grow between 5 to 10 meters tall. Its fruit consists of red, globular or ovoid capsules that grow in clusters resembling reddish-brown pods. Each capsule contains about 30 to 45 cone-shaped seeds, encased in a thin, waxy, blood-red aril. Once mature and dried, the capsules split open to reveal the vibrant seeds. For more than 200 years, *Bixa orellana* seeds and their extracts have been utilized as natural food coloring in North America and Europe. The seeds are particularly rich in flavonoids and carotenoids, making them a promising candidate for the green synthesis and stabilization of metal nanoparticles (Hussain et al., 2019). To the best of our knowledge, no prior studies have reported the synthesis of silver nanoparticles using the bioactive seed extract of *Bixa orellana*. This work presents, for the first time, a rapid and room-temperature method for synthesizing AgNPs using this unique plant-based extract.

2. MATERIALS AND METHODS

2.1 Materials

Silver nitrate (AgNO_3 , 99.9% purity) was purchased from Sigma-Aldrich. All glassware was thoroughly washed with distilled water and dried in an oven before use. Ultrapure water was used throughout the experiments. Fresh *Bixa orellana* seeds were obtained from local markets and authenticated by botanists at the Department of Botany.

2.2 Preparation of *Bixa orellana* Seed Extract

Fresh *Bixa orellana* seeds (50 g) were thoroughly washed with distilled water to remove any impurities and air-dried at room temperature. The cleaned seeds were then ground into a fine powder using a sterile mortar and pestle. Twenty grams of

the seed powder were added to 200 mL of deionized water and boiled for 30 minutes at 80°C. The extract was cooled to room temperature and filtered using Whatman No. 1 filter paper. The filtrate was stored at 4°C for further use in the synthesis of silver nanoparticles.

2.3 Green Synthesis of Silver Nanoparticles

For the synthesis of silver nanoparticles, 10 mL of the *Bixa orellana* seed extract was added dropwise to 90 mL of 1 mM silver nitrate (AgNO_3) solution with continuous stirring at room temperature. The reaction mixture was monitored for color change from yellowish-brown to dark brown, which indicated the formation of silver nanoparticles. The reduction process was allowed to proceed for 4 hours under ambient conditions. The colloidal solution was then centrifuged at 12,000 rpm for 20 minutes, and the resulting pellet was washed three times with deionized water to remove any unreacted silver ions and biomolecules. The purified AgNPs were dried in an oven at 60°C for 24 hours and stored in airtight containers for further characterization and biological activity studies. This methodology is similar to that reported by (Chondhe et al.2024) but adapted specifically for *Bixa orellana* seed extract.

2.4 Characterization of Silver Nanoparticles

2.4.1 UV-Visible Spectroscopy

The formation of silver nanoparticles was monitored using UV-visible spectroscopy in the wavelength range of 300-700 nm at a resolution of 1 nm. The measurements were taken at different time intervals (0, 30, 60, 120, 180, and 240 minutes) to monitor the progression of the reaction.

2.4.2 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was performed to identify the potential biomolecules in the *Bixa orellana* seed



extract responsible for the reduction and stabilization of the silver nanoparticles. The FTIR spectra of both the seed extract and the purified AgNPs were recorded using a Perkin Elmer Spectrum Two FTIR spectrometer in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1} .

2.4.3 X-Ray Diffraction (XRD) Analysis

The crystalline nature and phase identification of the synthesized AgNPs were analyzed using X-ray diffraction (Rigaku SmartLab) with $\text{Cu K}\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$) operating at 40 kV and 30 mA. The diffraction patterns were recorded in the 2θ range of 20° to 80° with a step size of 0.02° and a scan rate of 2° per minute.

2.4.4 Field Emission Scanning Electron Microscopy (FESEM)

The morphology and size of the silver nanoparticles were examined using FESEM (JEOL JSM-7600F) operating at an accelerating voltage of 15 kV. Prior to analysis, a thin layer of the powdered sample was spread on a carbon tape and sputter-coated with a thin layer of gold to make the sample conductive.

2.4.5 Transmission Electron Microscopy (TEM)

TEM analysis was carried out using a JEOL JEM-2100F microscope operating at an accelerating voltage of 200 kV. A drop of the AgNP suspension was placed on a carbon-coated copper grid and allowed to dry at room temperature before imaging.

2.4.6 Dynamic Light Scattering (DLS)

The hydrodynamic size distribution and zeta potential of the silver nanoparticles were measured using a Malvern Zetasizer Nano ZS90. The measurements were performed at 25°C with a scattering angle of 90° . The samples were

appropriately diluted with deionized water and sonicated for 15 minutes before analysis.

2.5 Biological Activity Evaluation

2.5.1 Antioxidant Activity

The antioxidant activity of the synthesized AgNPs was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Different concentrations of AgNPs (10, 20, 30, 40, and 50 $\mu\text{g/mL}$) were prepared in methanol. A 0.1 mM DPPH solution in methanol was prepared, and 1 mL of this solution was added to 3 mL of different concentrations of the AgNP solution. The mixture was incubated in the dark for 30 minutes at room temperature, and the absorbance was measured at 517 nm using a UV-visible spectrophotometer. Ascorbic acid was used as a positive control. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample.

2.5.2 Anticancer Activity

The in vitro anticancer activity of the synthesized AgNPs was assessed against human breast cancer (MCF-7), lung cancer (A549), and normal human embryonic kidney (HEK-293) cell lines using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37°C . The cells were seeded in 96-well plates at a density of 5×10^3 cells per well and allowed to attach overnight. The cells were then treated with different concentrations of AgNPs (5,



10, 25, 50, and 100 µg/mL) for 24 hours. After the treatment period, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37°C. The formazan crystals formed were dissolved in 100 µL of DMSO, and the absorbance was measured at 570 nm using a microplate reader. The cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = (\text{Absorbance of treated cells} / \text{Absorbance of control cells}) \times 100$$

2.5.3 Antibacterial Activity

The antibacterial activity of the synthesized AgNPs was evaluated against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains using the disk diffusion method, following a protocol similar to that described by (Chondhe et al. 2024). The bacterial strains were cultured in nutrient broth and incubated at 37°C for 24 hours. The bacterial suspensions (1.5×10^8 CFU/mL, equivalent to 0.5 McFarland standard) were spread on Mueller-Hinton agar plates. Sterile filter paper disks (6 mm diameter) impregnated with different concentrations of AgNPs (25, 50, 75, and 100 µg/mL) were placed on the inoculated plates. Ampicillin (10 µg/disk) was used as a positive control, and sterile distilled water was used as a negative control. The plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured in millimeters.

2.5.4 Antiproliferative Activity

The antiproliferative activity of the synthesized AgNPs was assessed using the colony formation assay. MCF-7 and A549 cells were seeded in 6-well plates at a density of 500 cells per well and allowed to attach overnight. The cells were then treated with different concentrations of AgNPs (5, 10, 25, and 50 µg/mL) for 24 hours. After the treatment period, the media containing the AgNPs was removed, and the cells were washed with PBS.

Fresh media was added, and the cells were allowed to grow for 14 days, with media changes every 3 days. The colonies formed were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.1% crystal violet for 30 minutes. The plates were washed with distilled water and air-dried. The number of colonies (>50 cells) was counted using a stereomicroscope, and the percentage of colony formation was calculated relative to the untreated control.

2.6 Statistical Analysis

All experiments were performed in triplicate, and the results are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad Prism 8.0 software. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine statistical significance. A p-value of <0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Visual Observation and UV-Visible Spectroscopy

The formation of silver nanoparticles was initially confirmed by the color change of the reaction mixture from yellowish-brown to dark brown, which is a characteristic indicator of AgNP formation due to the excitation of surface plasmon resonance (SPR). UV-visible spectroscopy analysis revealed a strong absorption peak at 420 nm, which is characteristic of the SPR phenomenon in silver nanoparticles. The intensity of the absorption peak increased with reaction time, reaching maximum intensity after 4 hours, indicating the complete reduction of silver ions to silver nanoparticles. Similar observations have been reported in previous studies using different plant extracts for AgNP synthesis (Shameli et al., 2012).

3.2 FTIR Analysis



FTIR analysis was performed to identify the potential biomolecules in the *Bixa orellana* seed extract responsible for the reduction and stabilization of the silver nanoparticles. The FTIR spectrum of the seed extract showed characteristic absorption bands at 3420 cm^{-1} (O-H stretching vibrations of phenolic and hydroxyl groups), 2922 cm^{-1} (C-H stretching vibrations), 1635 cm^{-1} (C=O stretching vibrations of amide groups), 1384 cm^{-1} (C-N stretching vibrations), and 1055 cm^{-1} (C-O-C stretching vibrations). The FTIR spectrum of the synthesized AgNPs showed shifts in the absorption bands compared to the seed extract, with peaks at 3380 cm^{-1} , 2918 cm^{-1} , 1622 cm^{-1} , 1375 cm^{-1} , and 1048 cm^{-1} . These shifts in the absorption bands suggest the involvement of hydroxyl, carbonyl, and amide groups in the reduction of silver ions and the stabilization of the resulting nanoparticles. The presence of these functional groups in the AgNP spectrum confirms that the phytochemicals from the *Bixa orellana* seed extract act as reducing and capping agents in the synthesis process, which aligns with findings reported by (Ahmed et al. 2016) for other plant extracts.

3.3 XRD Analysis

The crystalline nature and phase identification of the synthesized AgNPs were analyzed using X-ray diffraction. The XRD pattern revealed four distinct diffraction peaks at 2θ values of 38.1°, 44.2°, 64.6°, and 77.5°, corresponding to the (111), (200), (220), and (311) crystalline planes of the face-centered cubic (FCC) structure of silver, respectively. These peaks match well with the standard data for silver (JCPDS File No. 04-0783), confirming the crystalline nature of the synthesized AgNPs. The absence of additional peaks indicates the high purity of the synthesized nanoparticles. Similar XRD patterns have been observed in previous studies (Khan et al., 2019). The average crystallite size of the AgNPs was calculated using the Debye-Scherrer equation:

$$D = K\lambda / (\beta \cos \theta)$$

where D is the crystallite size, K is the Scherrer constant (0.9), λ is the X-ray wavelength (1.5406 Å), β is the full width at half maximum (FWHM) of the diffraction peak, and θ is the diffraction angle. The average crystallite size of the AgNPs was found to be 18.5 nm.

3.4 Morphological Analysis

3.4.1 FESEM Analysis

FESEM analysis revealed that the synthesized AgNPs were predominantly spherical in shape with a smooth surface morphology. The particles appeared to be well-dispersed with minimal aggregation, which could be attributed to the effective capping and stabilization by the phytochemicals present in the *Bixa orellana* seed extract. The size of the nanoparticles ranged from 10 to 30 nm, with an average size of 22 nm. These results are comparable to those reported by Patra, J. K., (2024) for ginger extract-mediated AgNP synthesis.

3.4.2 TEM Analysis

TEM analysis provided higher resolution images of the AgNPs, confirming their spherical shape and nanoscale dimensions. The TEM micrographs showed that the particles were well-dispersed and had a uniform size distribution. The average size of the AgNPs was found to be 20 nm, which is in good agreement with the XRD results. The selected area electron diffraction (SAED) pattern exhibited concentric rings with bright spots, further confirming the crystalline nature of the synthesized AgNPs.

3.5 DLS Analysis

Dynamic light scattering analysis revealed that the hydrodynamic size of the AgNPs ranged from 25 to 40 nm, with an average size of 35 nm. The larger hydrodynamic size compared to the TEM and



XRD results can be attributed to the presence of a biomolecular corona formed by the phytochemicals from the seed extract around the nanoparticles. The zeta potential of the AgNPs was found to be -28.6 mV, indicating good stability and dispersion of the nanoparticles in aqueous solution. (Xulu et al. 2022) have reported similar findings for AgNPs synthesized using other plant extracts.

3.6 Biological Activity Evaluation

3.6.1 Antioxidant Activity

The DPPH radical scavenging assay revealed that the synthesized AgNPs exhibited significant antioxidant activity in a concentration-dependent manner. The percentage of DPPH radical scavenging activity increased from 32.4% at 10 µg/mL to 78.9% at 50 µg/mL. The IC₅₀ value (the concentration required to scavenge 50% of the DPPH radicals) of the AgNPs was found to be 28.5 µg/mL, compared to 15.2 µg/mL for ascorbic acid, which was used as a positive control. The antioxidant activity of the AgNPs can be attributed to the presence of phytochemicals from the Bixa orellana seed extract, particularly flavonoids and phenolic compounds, which are known for their radical scavenging properties (Hussain et al., 2019).

3.6.2 Anticancer Activity

The MTT assay revealed that the synthesized AgNPs exhibited significant cytotoxicity against MCF-7 and A549 cancer cell lines in a concentration-dependent manner, while showing minimal toxicity towards normal HEK-293 cells. The IC₅₀ values for MCF-7 and A549 cells were found to be 32.7 µg/mL and 41.5 µg/mL, respectively, after 24 hours of treatment. In contrast, the IC₅₀ value for HEK-293 cells was >100 µg/mL, indicating the selective cytotoxicity of the AgNPs towards cancer cells. The selective anticancer activity of the AgNPs can be attributed to the differences in cellular uptake and

intracellular mechanisms between cancer and normal cells. Cancer cells are known to have a higher metabolic rate and increased endocytosis compared to normal cells, which can lead to enhanced uptake of nanoparticles. Additionally, the AgNPs might induce oxidative stress and DNA damage in cancer cells, leading to apoptosis (Patra et al., 2008).

3.6.3 Antibacterial Activity

The disk diffusion assay demonstrated that the synthesized AgNPs exhibited significant antibacterial activity against both Gram-positive and Gram-negative bacterial strains. The zones of inhibition increased with increasing concentrations of AgNPs. At a concentration of 100 µg/mL, the zones of inhibition were 18.5 mm for *S. aureus*, 16.2 mm for *B. subtilis*, 22.4 mm for *E. coli*, and 20.8 mm for *P. aeruginosa*. These results are comparable to those reported by Chondhe et al. (2024) for ginger extract-mediated AgNPs against similar bacterial strains. The stronger antibacterial activity against Gram-negative bacteria compared to Gram-positive bacteria can be attributed to the differences in their cell wall structures. Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane composed of lipopolysaccharides, which may facilitate the penetration of AgNPs. The antibacterial mechanism of AgNPs involves multiple pathways, including disruption of bacterial cell membranes, generation of reactive oxygen species (ROS), and interference with DNA replication and protein synthesis (Nakamura et al., 2019).

3.6.4 Antiproliferative Activity

The colony formation assay revealed that the synthesized AgNPs exhibited significant antiproliferative activity against MCF-7 and A549 cancer cells in a concentration-dependent manner. The percentage of colony formation decreased from 85.6% at 5 µg/mL to 21.3% at 50 µg/mL for



MCF-7 cells, and from 89.2% at 5 µg/mL to 28.7% at 50 µg/mL for A549 cells. The IC₅₀ values (the concentration required to reduce colony formation by 50%) were found to be 24.8 µg/mL for MCF-7 cells and 30.2 µg/mL for A549 cells. The antiproliferative activity of the AgNPs can be attributed to their ability to induce cell cycle arrest and inhibit key cellular processes involved in proliferation. The AgNPs might interfere with signaling pathways that regulate cell cycle progression, leading to cell cycle arrest and inhibition of proliferation. Additionally, the ROS generated by the AgNPs can cause oxidative damage to cellular components, further contributing to their antiproliferative effects (Strohal et al., 2005).

4. CONCLUSION

In this study, we have successfully synthesized silver nanoparticles using an aqueous seed extract of *Bixa orellana* through a simple, eco-friendly, and cost-effective green synthesis approach. The formation of AgNPs was confirmed by UV-visible spectroscopy, which showed a characteristic SPR peak at 420 nm. FTIR analysis revealed the involvement of phytochemicals from the seed extract in the reduction and stabilization of the AgNPs. XRD analysis confirmed the crystalline nature of the AgNPs, while FESEM and TEM analyses demonstrated their spherical morphology and nanoscale dimensions. DLS analysis indicated good stability and dispersion of the nanoparticles in aqueous solution. The biosynthesized AgNPs exhibited significant antioxidant, anticancer, antibacterial, and antiproliferative activities, suggesting their potential applications in various biomedical fields. The selective cytotoxicity of the AgNPs towards cancer cells while showing minimal toxicity towards normal cells makes them promising candidates for cancer therapy. Similarly, their strong antibacterial activity against both Gram-positive and Gram-negative bacteria suggests their potential use as antimicrobial agents. This study highlights the potential of *Bixa*

orellana seed extract as a natural reducing and stabilizing agent for the green synthesis of silver nanoparticles with enhanced biological activities. The simple, rapid, and room-temperature synthesis method described in this work offers a sustainable alternative to conventional physical and chemical synthesis methods, aligning with the principles of green chemistry. Future studies should focus on elucidating the molecular mechanisms underlying the biological activities of these nanoparticles and exploring their potential applications in drug delivery systems, wound healing, and other biomedical fields. Additionally, in vivo studies are needed to evaluate the safety and efficacy of these nanoparticles for potential clinical applications.

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