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Green synthesis of silver nanoparticles using *Annona muricata* extract and their antibacterial and antibiofilm activity against multidrug-resistant bacteria

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ABSTRACT

The study centered on the synthesis, characterization, and application of silver nanoparticles (Ag NPs), which show potential for biomedical applications. Nanobiotechnology allows for broad NP application while reducing the reliance on expensive and hazardous chemicals through the sustainable production of NPs from plant resources. This research aimed to develop an environmentally safe process for Ag NPs using the Annonaceae fruit's aqueous extract as a capping and reducing agent and assess their potential pharmaceutical applications as antibacterial and antifungal agents. Ag NPs were characterized via ultraviolet–visible spectroscopy technique, Fourier transform infrared spectroscopy, field-emission scanning electron microscopy, atomic force microscopy, and X-ray diffraction. The biological activity of Ag NPs was investigated against two genera of pathogenic bacteria, namely, Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*, through the agar well diffusion assay. This study included the plant's possible use in the pharmaceutical industry. The plant's nutritious fruit extract opens the door to further the research on its possible application as an edible medicine.

1. INTRODUCTION

Nanotechnology, an incredible and developing technological innovation, has captivated the curiosity of experts from various disciplines. Unique qualities created at nanoscale levels are made possible by nanoparticles (NPs), which have many uses in chemical engineering, biomedicine, pharmacology, and agriculture [1][2]. An emerging field in nanoscience and nanotechnology is the application of materials structures at nanoscale measurements, typically between 1 and 100 nm. In biology, biomedical sciences, water treatment, solar energy conversion, catalysis, and biomaterials, nanomaterials may offer answers to technical and environmental problems [3].

Plant extracts are utilized in the green synthesis of NPs and offer an alternative solution to traditional chemical and physical processes. The corresponding method is simple, reliable, and safe and provides an environmentally friendly and nontoxic approach [4].

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Biological agents use naturally existing proteins, enzymes, and phytochemicals to produce and cap nanohybrids; chemical and physical approaches require considerable energy and chemicals to synthesize NPs [5]. Plants provide natural ingredients that have been extensively utilized in the pharmaceutical industry[6]. *Annona muricata* belongs to the Annonaceae family, which comprises 130 genera and 2300 subspecies. This plant is grown in tropical and subtropical areas, such as South America, Southeast Asia, Australia, and the rainforests of Africa[6][7]. Leaves, stems, and fruits of the *Annona muricata* plant have been used to treat various human diseases, including various types of cancers and pathogenic microorganisms [7]. Green chemistry has improved the purification and sensitivity of certain substances utilized in anticancer and antibacterial therapies[8][9][10][11][12]. The production of biological silver NPs (Ag NPs) is easy, direct, reliable, nontoxic, and environmentally friendly [13]. The generation of Ag NPs using green compounds is reliable and biocompatible with other medical applications. The wide biological activity of Ag NPs has enabled its application in disinfectant sprays, textiles, antibacterial agents, and wound dressings [14].

Annona muricata is a member of the *Annonaceae* family and inhabits tropical and subtropical

regions around the world. This tree can grow up to a height of 5–8 m, with diameters of approximately 15–83 cm. Its canopy is open and round, and it has relatively large, shiny leaves that are dark green in color[15]. In established traditional medicine, many parts of the plant are used to treat parasites, the plague, diabetes, high blood pressure, and infection. The fruits and seeds are applied in the treatment of fever and arthritis and in eliminating worms[16].

Ag NPs exhibit unique physical, chemical, and biological qualities. Notably, they have been utilized as antibacterial and antifungal agents[17][18]. The antibacterial effect of Ag NPs is achieved through various processes, including the disruption of bacterial cell membranes, inhibition of enzyme activity, and generation of reactive oxygen species [19]. Similarly, the simultaneous presence of phytochemical substances found in plant extracts and Ag NPs promotes the growth of antibacterial activity and prevents the emergence of bacterial resistance [20].

The provision of alternative medicines stems from growing concern regarding antimicrobial drug resistance, which is one of the most important public health risks. A promising solution to this issue can be the biosynthesis of NPs as a new class of antimicrobial medicines. This study aimed to develop a facile and ecofriendly method for the synthesis of Ag NPs from *Annona muricata* fruit extract and evaluate their biological effect as antibacterial and antibiofilm.

.2. Materials and methods

2.1 Materials

All analytic chemical materials were purchased from Sigma-Aldrich Co. *Annona muricata* and silver nitrate ($AgNO_3$) were supplied by Sigma Chemicals Company (St. Louis, Missouri, USA). The deionized water (H_2O, Belgium) was supplied by Chem-Phi Nanoscience Center, Baghdad.

2.2 Methods

2.2.1 Preparation of *Annona muricata* fruit extract

A total of 100 mL distilled water was heated until 60 °C. Then, 3 g dried seedless *Annona muricata* fruit powder was added with constant stirring for 1 h. The product was then filtered using Whatman Filter Paper No. 1, and an aqueous brown *Annona muricata* fruit solution was generated.

2.3 Synthesis of Ag NPs

To prepare the Ag NPs, we weighed 0.1 g $AgNO_3$ in (50 mL) distilled water and combined it with 1 mL *Annona muricata* fruit aqueous extract. After continuous washing of the particles, Ag NP formation was confirmed by the alteration of the color to dark brown and centrifugation of the particles for 25 min at 12,000 rpm. Ag NPs were repeatedly rinsed with deionized water to remove untreated reaction mixtures. Next, the biological effect and physical–chemical characteristics of Ag NPs were assessed (Figure 1).

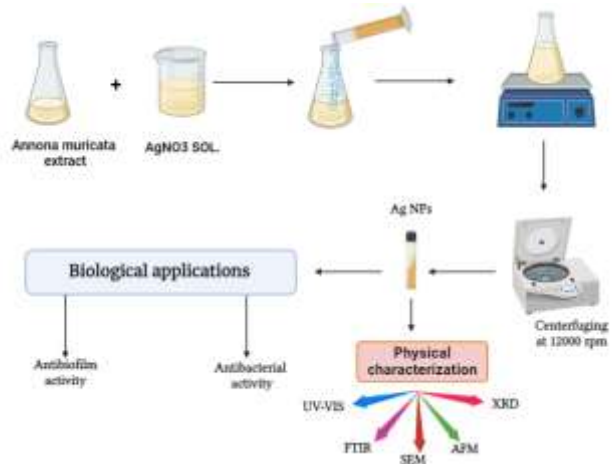

Figure 1: Synthesis of Ag NPs

2.4 Characterization of Ag NPs

The absorption spectrum of Ag NPs was measured at the wavelength range of 200–600 nm using a T80 UV/VIS spectrophotometer. Fourier transform infrared (FT-IR) spectroscopy was performed to identify biomolecules that effectively stabilized and capped Ag NPs. Field-emission scanning electron microscopy (FESEM) (Carl Zeiss (Germany) and atomic force microscopy (AFM) were utilized to determine the size and shape of Ag NPs, respectively. The crystalline structure of Ag NPs was further assessed using an automated diffraction meter and X-ray diffraction (XRD) (Shimadzu 6000 XRD) [21] (Figure 1).

2.5 Biological Applications of Ag NPs

2.5.1 Antibacterial activity

The antibacterial potential of Ag NPs was examined against *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* via an agar well diffusion assay[22][23]. Muller–Hinton agar (20 mL) was aseptically added to sterile Petri dishes. The bacterial species were extracted from their stock cultures using a sterile wire loop. Then, 6 mm-diameter wells were bored onto the agar plates using a sterile point following the culture of organisms. A total of 12.5, 25, 50, and 100 µg/mL Ag NPs were added to the bored wells. The cultured plates containing Ag NPs, *S. aureus*, and *E. coli*

were incubated at 37 °C for 24 h before the inhibitory zone diameter average was measured and recorded [24][25].

2.5.2 Antibiofilm

The antibiotic activity of Ag NPs against bacterial strains was estimated through implementation of a previously described methodology [26]. In a six-well plate, *E. coli* and *S. aureus* were cultured at a temperature of 1 * 10 6 and kept for 24 h at 37 °C. After the removal of the remaining suspended bacterial cells from each well of the microtiter plate, the plate was left to dry for 1 h at 37 °C. To study and evaluate the effect of Ag NPs as an antibiofilm agent, we injected 100 µg/mL Ag NPs into the wells and left them for another 24 h at 37 °C. The samples were washed once with phosphate-buffered saline, and the wells were left to dry for 45 min. Next, the bacteria adhering to the wells were stained using 0.1% crystal violet. The plate was then stored for 30 min at 37 °C in the dark, followed by a single wash with ethanol (95%). An enzyme-linked immunosorbent assay reader for microplates was used to measure the drop in biofilm biomass at 595 nm. The percentage of biofilm biomass removal was computed using the following formula:

Biofilm elimination % = A/B*100

where A refers to the optical density $(OD)_{595}$ of untreated control - OD_{595} of Ag NP-treated biofilm; B refers to the OD_{595} of the untreated control

2.5.3 Statistical analysis

The data were obtained and statistically analyzed using GraphPad Prism version 5.01 [27]. The data were provided as the mean ± standard error of triplicate measurements. One-way analysis of variance (ANOVA) with Dunnett's post-test was performed to indicate statistically important differences, with $p<0.05$ regarded as significant [28]

3. RESULTS AND DISCUSSION

3.1 Characterization of Ag NPs

3.1.1 Ultraviolet–visible (UV-Vis) spectroscopic analysis

Ag NPs were verified using a UV-Vis spectrophotometer. The absorption maximum was observed at wavelengths ranging from 200 nm to 600 nm. A broad absorption peak of Ag NPs was detected at a wavelength of 445 nm (Figure 2). In this context, a previous study revealed the peak absorption of Ag NPs at 440 nm [29].

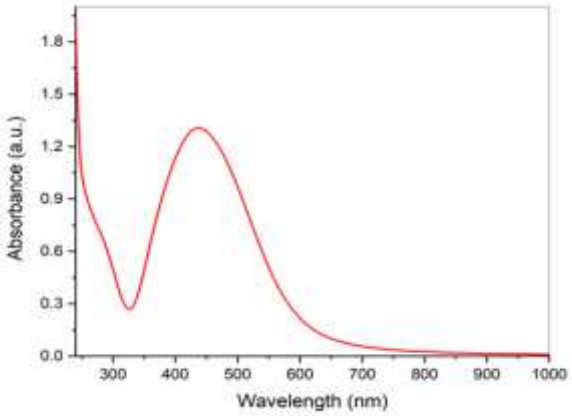

Figure. 2: UV–Vis spectrum of Ag NPs

3.1.2 FT-IR

FT-IR measurements identified biomolecules that effectively stabilized and capped the Ag NPs synthesized by *Annona muricata*. Figure 3 shows the spectrum obtained in the FTIR analysis of Ag NPs. The band at 3339 cm^{-1} was compatible with O-H stretching H-bonded alcohol and phenols. The band at 1627 cm^{-1} was compatible with N-H bend primary amines. The peak at 1379 cm^{-1} corresponded to the C-N stretching of the aromatic amine group, and the bands observed at 1150, 1067, and 1020 cm^{-1} were compatible with C-N stretching of alcohols, carboxylic acids, ethers, and esters, respectively. Therefore, the synthesized NPs can be enclosed by proteins and metabolites, such as terpenoids with functional groups of alcohols, ketones, aldehydes, and carboxylic acids. Our findings were consistent with those of a previous study [29].

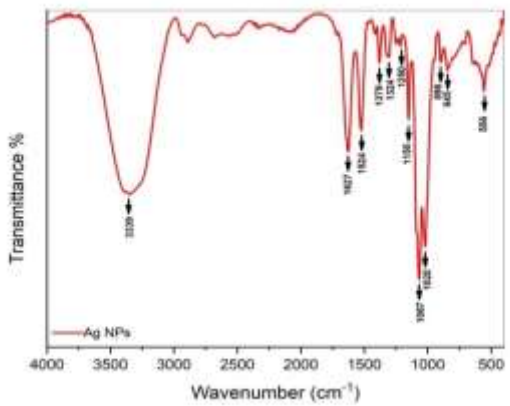

Figure. 3: FT-IR spectrum of Ag NPs

3.1.3 SEM

During SEM, a high-energy electron beam was

used to scan and produce a sample image. The interaction between the electrons and atoms in the sample yielded various signals, such as secondary electrons and back-scattered X-rays[30]. Figure 4 depicts the shape and size of the Ag NPs generated from *Annona muricata*, with the FESEM image revealing spherical pure silver particles with a diameter of 89.32 nm. Previous studies showed that the Ag NPs manufactured using *Caralluma flava* and *Nigella sativa* extracts were spherical [31][32].

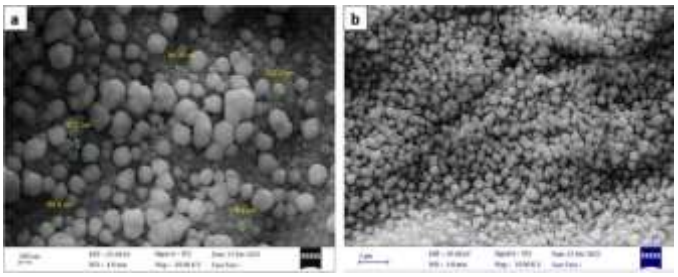

Figure 4: SEM images of Ag NPs (a) 100 nm and (b) 1 µm

3.1.4AFM

AFM was used to assess the characterization of Ag NPs (Figure 5). The synthesized Ag NPs were visualized in two-dimensional (2D) and 3D forms. The images showed the dispersal of Ag NPs, whose average diameter reached 57.40 nm.

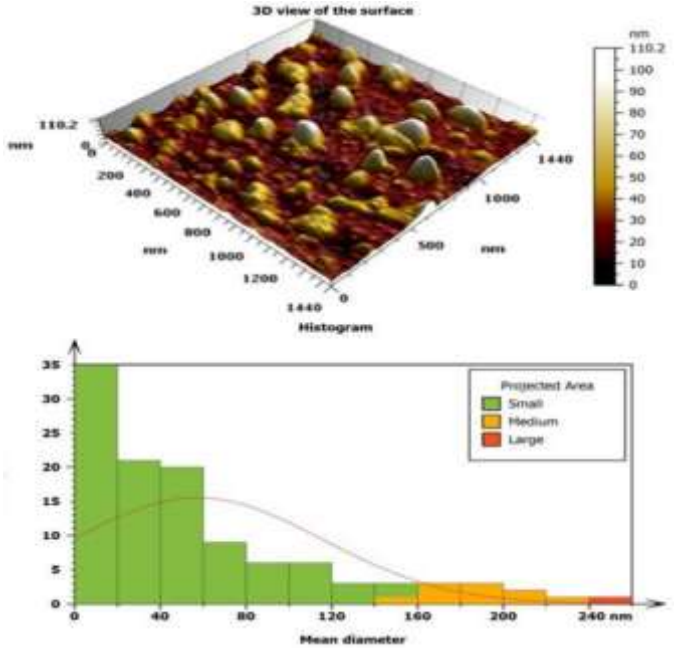

Figure 5: AFM images of 3D structure and average distribution

3.1.5 XRD

Figure 6 shows the XRD pattern of pure Ag NPs with the crystalline structure mediated by *Annona muricata* extract. The XRD pattern was indexed to a standard reference database called the Joint Committee on Powder Diffraction Standards data file card No. 01-080-1269. The Ag NPs were distinguished by their distinctive diffraction peaks at (2θ) 29.48° and 32.42°, which correlated to the 110 and 111 planes of the face-centered cubic (fcc) structure [33]. The XRD pattern from a previous study demonstrated the fcc crystalline structure of Ag NPs derived from *Allophylus cobbe* [34]. The result of the current study is consistent with that of a recent research[35].

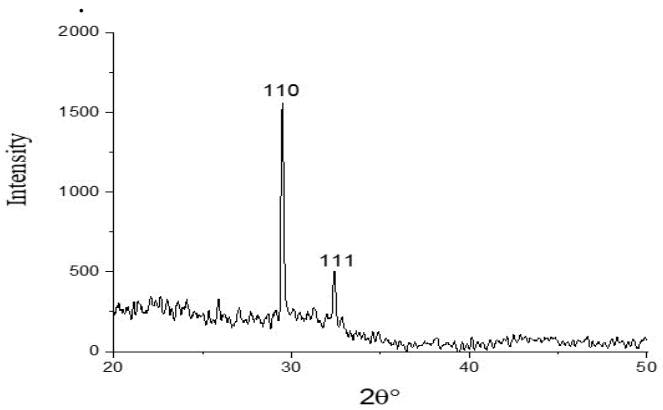

Figure 6: XRD pattern of Ag NPs

3.2. Antibacterial activity of Ag NPs

The antibacterial efficacy of Ag NPs was examined against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive), in an agar well diffusion experiment. The Ag NPs exhibited a substantial efficacy against *E. coli* and *S. aureus* in a concentration-dependent manner. At the highest concentration (100 µg/mL), the Ag NPs exhibited a significant inhibition against *E. coli* and *Staphylococcus aureus*, with average inhibition diameters of 25 and 19.3 mm, respectively. In addition, the average inhibition diameters were 19 and 17 mm at the lowest concentration. Distilled water served as a negative control. Table 1 and Figure 7 indicate the inhibition zones of Ag NPs against the studied bacterial strains.

The antimicrobial efficacy of Ag NPs is primarily linked to their capability to cause structural deformities within bacterial cell membranes. Moreover, the minimal dimensions and spherical morphology of these NPs contributed to an increased surface area. This amplification in surface area enhanced their interaction with the bacterial membrane, which led to a more

effective induction of membrane failure in the targeted bacteria[31][36]. Contemporary research demonstrated that the Ag NPs derived from the fenugreek plant effectively exterminated bacterial cells. This bactericidal action occurred as a result of protein leakage, which was observed within 4 h of exposure, and attributed to the enhanced permeability of the bacterial cell membrane induced by NPs [37]. Another study reported that the Ag NPs prepared from Grewia Tenax plant extract exhibited a significant activity against Gram-positive *S. aureus* and Gram-negative *E. coli* [38].

Table 1: Antibacterial activities of Ag NPs against *E. coli* and *S. aureus*

Ag NPs (µg/mL)	The mean diameter of inhibition zone (mm) / *E. coli*	The mean diameter of inhibition zone (mm) / *S. aureus*
12.5	19 ± 0.30	17 ± 0.50
25	21 ± 0.50	17.5 ± 0.40
50	23 ± 0.50	18 ± 0.50
100	25 ± 1.00	19.3 ± 0.5

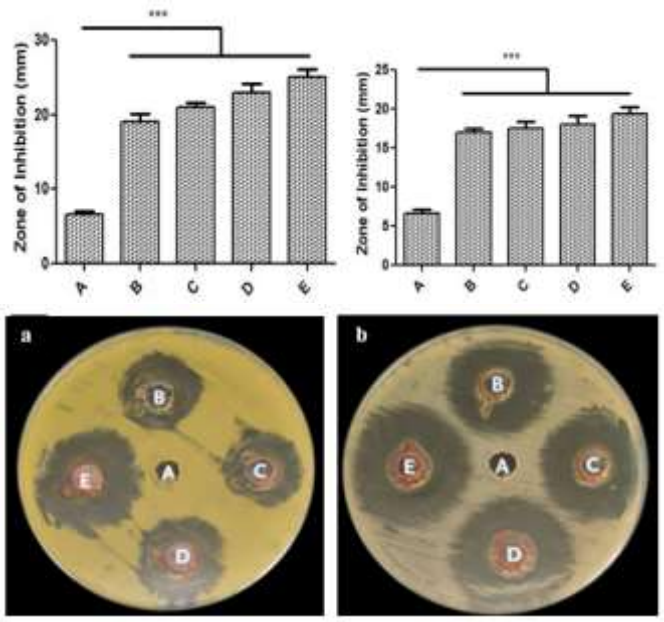

Figure 7: (a) Antibacterial activity of Ag NPs against *E. coli*. A, control (Distilled water). B, 12.5 µg/mL. C, 25 µg/mL. D, 50 µg/mL. E, 100 µg/mL. (b) Antibacterial activity of Ag NPs against *S. aureus*. A, Control. B, 12.5 µg/mL. C, 25 µg/mL. D, 50 µg/mL. E, 100 µg/mL

3.3 Antibiofilm activity of Ag NPs

In the current study, in vitro conditions were used to inspect the dose-dependent capability of Ag NPs to suppress the activity of biofilms generated by the human pathogens *E. coli* and *S. aureus*. These bacterial strains were initially investigated to create a biofilm. After being cultivated in microtiter plate wells for 24 h, the bacterial strains were treated with Ag NPs (100 µg/mL). Figure 8 shows that treatments with 100 µg/mL Ag NPs reduced the biofilm biomass in *E. coli* and *S. aureus* by 56% (***p < 0.001) and 18% (*p < 0.05), respectively. A previous in vitro study revealed that 24 h treatment of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* with Ag NPs caused more than 95% suppression in biofilm formation[39]. A recent study also revealed that the Ag NPs prepared from *Momordica charantia* extract showed a significant antibiofilm activity against *Aeromonas hydrophilia* and *Enterococcus faecalis* [40].

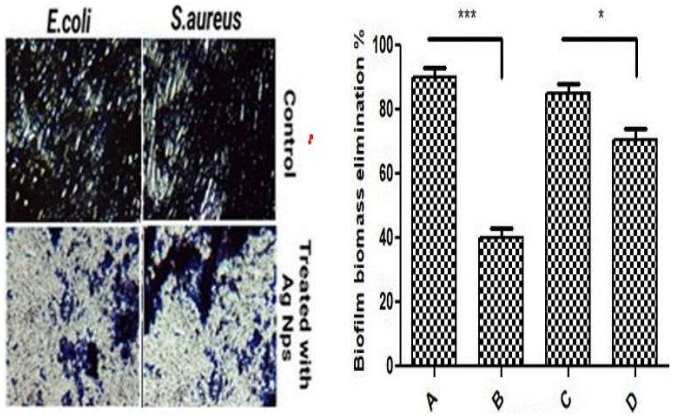

Figure 8: Biofilm biomass reduction in *E. coli* and *S. aureus* after treatments with 100 µg/mL Ag NPs. A) *E. coli*. B) *E. coli* + 100 µg/mL Ag NPs. C) *S. aureus*. D) *S. aureus* + 100 µg/mL Ag NPs. Each value is the mean ± standard error of the mean (n=3). The data were assessed via one-way ANOVA. *p < 0.05, ***p < 0.001 versus untreated cells

Conclusions

The present study reported a quick, easy, safe, cheap, and ecofriendly method for the preparation of Ag NPs using *Annona muricata* fruit extract. Following structural characterization, the Ag NPs were assessed as antibacterial and antibiofilm agents. Based on the findings of this study, the Ag NPs can be used to create effective antibacterial medications to combat increasingly resistant bacterial strains. However, further investigation of their mechanism of action and identification of extract components will allow for a broader range of biomedical uses.

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التوليف الأخضر لجسيمات الفضة النانوية باستخدام مستخلص Annona Muricata ونشاطها المضاد للبكتيريا والمضادات الحيوية ضد البكتيريا المقاومة للأدوية المتعددة

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الخلاصة:

تتمحور الدراسة حول تخليق وتوصيف وتطبيق جسيمات الفضة النانوية (Ag NPs)، والتي تمتاز بامكاناتها الواعدة التي يمكن توجيهها نحو التطبيقات الطبية والتطبيقات الحيوية. حيث يمكن ان توفر تقنية النانو الحيوية مجالات واسعة من التوظيفات مع تقليل الاعتماد على المواد الكيميائية التي يعاني الباحثون من اثمانها الباهظة فضلا عن خطورتها. ويكون ذلك من خلال الإنتاج المستدام للجسيمات النانوية من الموارد النباتية. يهدف هذا البحث إلى تطوير عملية امنة بيئيا لإنتاج جسيمات الفضة النانوية (Ag NPs) باستخدام المستخلص المائي لفاكهة *Annonaceae* كعامل تغطية واختزال، وتقييم تطبيقاتها الصيدلانية المحتملة كعوامل مضادة للبكتيريا ومضادة للفطريات. تم توصيف Ag NPs باستخدام تقنية التحليل الطيفي للأشعة فوق البنفسجية، الاشعة تحت الحمراءFT-IR ، المجهر الإلكتروني الماسح للانبعاث الميداني ((FESEM، AFM وحيود الأشعة السينية (XRD). تم دراسة النشاط البيولوجي - Ag NPs ضد جنسين من البكتيريا المسببة للأمراض، هما سالبة الجرام*Escherichia coli* و*Staphylococcus aureus* إيجابية الجرام، وذلك باستخدام اختبار الانتشارخلال الاكار بشكل جيد. تتضمن هذه الدراسة إمكانية استخدام النبات في صناعة الأدوية. يفتح استخدام مستخلص ثمار الفاكهة المغذية الباب لمزيد من البحث حول إمكانية استخدامه كدواء صالح للأكل.

الكلمات المفتاحية: الجسيمات النانوية الفضية،الكيمياء الخضراء ،القشدة الشائكه (الجرافيولا)،مضادات الميكروبات