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Use of *Citrullus colocynthis* Callus for Green Synthesis of Silver Nanoparticles and their Activity Against Biofilm-Producing

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I N F O

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A B S T R A C T

Introduction: The field of nanotechnology has developed in the recent past and its use has increased in the medical field. This study has attempted to analyse the activity of silver nanoparticles on biofilm formation in *Pseudomonas aeruginosa*.

Method: In this study, 30 bacterial samples were used to study the activity of silver nanoparticles on biofilm formation in *Pseudomonas aeruginosa*. The seeds of *Citrullus colocynthis* were grown in the laboratory and their leaves were used to produce callus using different hormone concentrations. The callus was grown and was used to produce silver nanoparticles. Ten bacterial isolates (3 strong, 4 medium, and 3 weak) were treated with silver particles to study their effect on biofilm production.

Results: The study showed variation in the ability of bacteria to produce biofilms. It was found that 11 (36.7%) samples had a high ability to form biofilms, 16 (53.3%) had a moderate ability to form biofilms, while the rest of the samples (3, 10%) showed a weak ability to form biofilms.

Conclusion: The study showed a decrease in biofilm production levels for all studied bacterial isolates. This indicated that silver nanoparticles may have the ability to inhibit or reduce biofilm production in *Pseudomonas aeruginosa* bacteria.

Keywords: Citrullus colocynthis, *Pseudomonas aeruginosa*, Silver Nanoparticles, Green Synthesis, Biofilm Formation

Introduction

Recently, nanotechnology has developed greatly and has been used in a wide range of applications to prevent, diagnose, and treat various diseases.[1,2] Nanoparticles have the advantage of being able to accumulate effectively due to their small size (from 1 to 100 nm), three-layer structure (surface layer, shell layer, and base layer), ability to encapsulate drugs, and increased surface-to-volume ratio. In addition, nanoparticles can penetrate a cell membrane.[3] Moreover, nanoparticles have a highly tunable property to bind to a variety of ligands, allowing them to be used effectively in various biological applications. Many types of nanoparticles have been developed, such as metallic (silver, platinum, gold, and palladium), magnetic, ceramic, lipid, and polymeric nanoparticles.[4] Silver nanoparticles (AgNPs) are widely used to design biomedical devices, wound dressings, and antimicrobial coatings.[5] Chemical deposition, hydrothermal, biological, and sol-gel processes, reverse micelle and hydrothermal methods are used for their synthesis. The green synthesis of AgNPs using plant extracts as a reducing agent is developing rapidly due to the extracts' non-toxic and cost-effective attributes.[6] Citrullus colocynthis (C. colocynthis) is a genetically diverse, widespread, drought-tolerant desert plant that belongs to the Cucurbitaceae family. In several studies, the biomedical aspects of C. colocynthis have been investigated, including its antimicrobial, anticancer, antioxidant, and lipid profile properties.[7,8] It is rich in various biomolecules, such as flavonoids, glycosides, fatty acids, phenols, and alkaloids.[9] These phytochemicals can act as bioreduction and stabilisation agents in the biosynthesis of AgNPs by converting silver ions (Ag^+) to free silver. The presence of phytochemicals on the surface of the nanoparticles could enhance the functions of AgNPs as biomedical agents due to their synergistic effects. Moreover, these bioactive entities can also catalyse redox reactions and act as stabilising agents for Cc-AgNP synthesis.[10]

The current study aimed to synthesise AgNPs using C. colocynthis fruit as a reducing agent and to investigate the effect of the particles on the biofilm synthesis of P. .

Materials and Methods

Plant Materials

C. colocynthis seeds obtained from the local market in Baghdad, Iraq were used in this study. They were classified by botanist Dr Sakina Abbas Aliwi in the Department of Biology, College of Science, University of Baghdad. The study duration was from May 2021 to May 2022.

The seeds were sterilised by immersing them in 70% ethanol for one minute after being cleaned for five minutes under running water. Seed sterilisation was performed inside a laminar air flow cabinet with immersion in 3% sodium hypochlorite and one drop of Tween-20 for ten minutes, and then washed several times in sterile distilled water.

Sterile seeds were transferred to a hormone-free Murashige and Skoog (MS) medium with previously prepared 3% sucrose. One seed was grown in each glass flask containing 10 ml of MS medium, incubated at 25 ± 2 °C for 24 h in the dark for 2 weeks. After germination, when seedlings achieved sufficient growth to be used for callus induction, C. colocynthis fresh leaves were gathered. Two-week-old C. colocynthis plants' leaves were infected in medium with different doses of 6-BA (0.1–0.8 mg/L) and 2,4-D (1–10 mg/L) to produce callus. In MS media, supplemented with 6-BA/ 2,4-D (0.4 mg/L/ 6 mg/L), leafy explants had a greater cholerogenic frequency (70%) than control samples. From the green base of the internodes, a compact green callus began to grow, and the entire plant became a callus mass.

Extract Preparation and Nanoparticles (AgNPs) Synthesis

A mortar and pestle was used to grind around 20 g of callus in 100 mL of sterile distilled water. A filter paper was used to separate the resultant extract before using it to create silver nanoparticles. A 90 ml aqueous solution of silver nitrate solution (1 mM) was added separately to 10 ml of callus culture suspension for reduction to Ag^+ ions, and the mixture was then incubated at room temperature (35 °C) for around 24 hours. By keeping an eye on how the medium's colour changed from green to dark brown, the initial identification of the synthesised silver nanoparticles in the reaction mixture was carried out. By repeatedly centrifuging the reaction mixture at 10,000 g for 10 min 4–5 times, silver nanoparticles were separated and concentrated. For optical measurements, the solution was maintained as a lyophilised powder and the supernatant was always refilled with distillate.

Biofilm Assay

In the current study, 30 clinical isolates of P. aeruginosa were screened for their capability to form biofilm via the microtitration plate method. After preparing sterilised Brain Heart Infusion broth with 2% sucrose, 180 µL of the broth was treated with silver nanoparticles added to each well. 20 µL of P. aeruginosa suspension was introduced compared to 0.5 MacFarland, whereas the control contained just 180 µL and 20 µL of P. aeruginosa suspension. After incubation, the medium was taken from the wells and washed three times with sterile phosphate-buffered saline (PBS) to remove the unattached P. aeruginosa cells. It was then left to dry for 15 minutes at room temperature. The wells were filled with 200 µL of crystal violet (0.1%) and allowed to sit for 20 minutes. To remove the unbound dye, the stained wells were washed three times with PBS (pH 7.2) and allowed to dry at room temperature for 15 minutes. Finally, 200 µL of 95% ethanol was poured into each well, and the optical

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density was measured using an Elisa reader at 630 nm.

Statistical Analysis

Graph Pad Prism 8.4.2 was used to conduct the independent t-test, one-way and two-way ANOVA, and Tukey's post hoc test for multiple comparisons. Data were deemed significant if the significance threshold (p) was 0.05 or less.

Results

Growing *Citrullus colocynthis* from Seeds

The seed germination rate of *Citrullus colocynthis* inoculated on MS medium was 100% (Figure 1). The findings of culture showed that shoots from apices and nodes were discharged in MS basal media after about a week. With a medium that had been altered with various benzylaminopurine concentrations, multicotyledon development was accomplished. The germination results demonstrated a modest swelling before the plant's appearance in the incubated nodes. After two weeks of culture, MS medium with BAP 4.0 mg/L produced the most shoots per node of any phytohormone concentration tested.

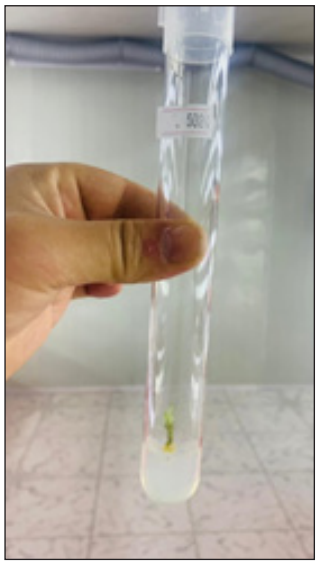

Figure 1.Growing *Citrullus colocynthis* from Seeds

Preparation of Silver Nanoparticles

When stem-derived callus extract was combined with a solution containing 1 mM silver nitrate, the reaction mixture became brown, indicating the formation of silver nanoparticles (Figure 2).

Figure 2.Appearance of the Reaction Mixture

Biofilm Formation

Thirty identified isolates of P. aeruginosa bacteria were obtained. Biofilm formation assay was used to evaluate the ability of the bacteria to form biofilms using a microtitration plate, as biofilms are among the most important virulence factors that bacteria possess which help them to increase resistance. The absorbance was determined by an ELISA device at a wavelength of 630 nm to ascertain the degree of biofilm formation of the studied isolates attached to the microtitration plate.

The ability to produce a biofilm was studied for samples before and after treatment with silver nanoparticles extracted from C. colocynthis callus at sub-lethal concentration (Figure 3).

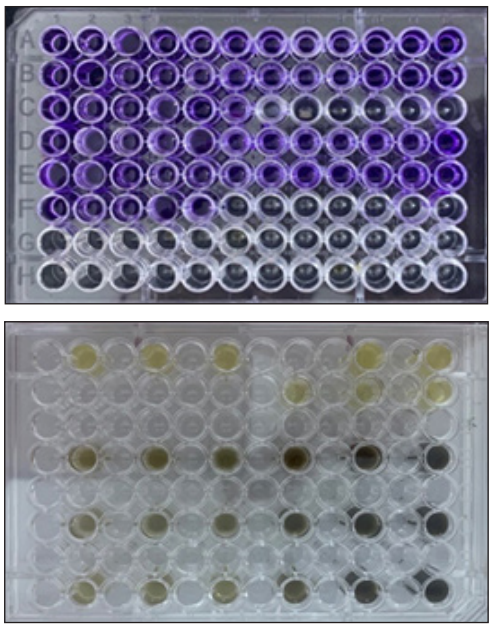

Figure 3.Ability to Produce a Biofilm Before and After Treatment with Silver Nanoparticles

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The results in Table 1 indicate the ability of bacteria to form biofilms. The percentage of biofilm formation was 37% for strong formation, 53% for medium formation, and 10% for weak formation.

Table 1.Ability of Bacteria to Produce Biofilms

Biofilms	Number	Percentage
Strong	11	36.7
Medium	16	53.3
Weak	3	10.0
Non-biofilm	0	0.0

Biofilm Formation Before and After Treatment with Silver Nanoparticles

Ten isolates were selected to test the effect of silver nanoparticles on biofilm formation, where 3 isolates were selected for strong biofilm formation, 4 for medium formation, and three for weak biofilm formation. These isolates were treated with a sub-lethal concentration of silver nanoparticles. The results of the study showed a decrease in the levels of biofilm production in all isolates treated with silver nanoparticles compared to the untreated isolates (Figure 4).

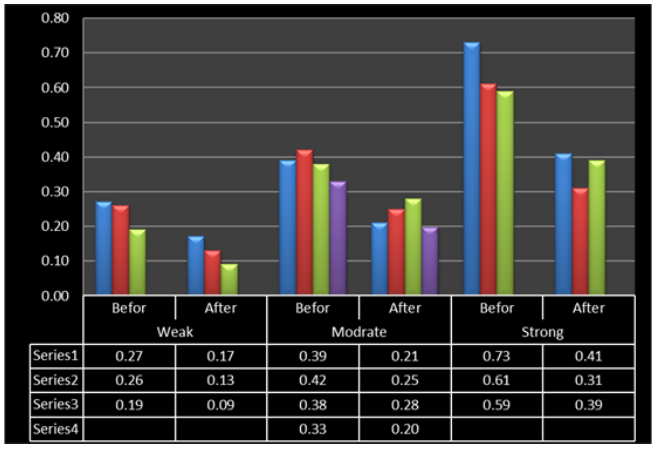

Figure 4.Biofilm Formations Before and After Treatment with Silver Nanoparticles

Discussion

Under sterile circumstances, in vitro germination increases the likelihood of seed germination and plantlet survival. The germination of seeds is aided because more moisture is available to the seeds than with a hard seed coat.[11] Many researchers have used MS basal media for in vitro seed germination of various plants.[12] Others who were experimenting with different MS media compositions discovered that 1/2 strength MS media was sufficient for an increase in germination percentage, root length, and shoot length.[13,14] MS medium is considered one of the most suitable culture media. Meena et al. experimented with several culture media on the hibiscus plant and found that the most suitable medium for the germination of hibiscus is the MS medium.[15] It is evident from the data that callus cultures are an excellent method for synthesising nanoparticles since they are formed under alkaline conditions and are suited for biomedical applications. Since the reaction in the current investigation was practically finished in two hours, the rate of reduction was found to be quite quick. The reaction was carried out using a simple synthetic approach at room temperature and 2–4 pH. This demonstrates the high potential for manufacture of silver nanoparticles in the callus tissue of C. colocynthis. Our findings were comparable to those previously reported for the use of Carcia papaya callus extract,[16] Capsicum annuum leaf extract,[17] and Aloe vera extract[18]. This demonstrates the tremendous potential of C. colocynthis callus cultures for the production of AgNPs. Therefore, by increasing the concentration of callus extract and appropriately altering the reaction conditions, it would be feasible to obtain an even quicker rate of AgNP synthesis. The callus is made up of meristematic cells that divide quickly and are highly capable of biosynthesising phytochemicals like phenolics, which may be essential for the production and stability of nanoparticles. Our findings concur with those for Sesuvium and Vigna radiata[19,20] which revealed that callus cultures rather than plant components could be used to quickly synthesise silver nanoparticles, and this was explained by the presence of rapidly proliferating cells and phytochemical-rich callus.

Hande et al. studied the ability of P. aeruginosa to form a biofilm according to the type of infection and the site from which the isolate was obtained. They found that the average biofilm composed of P. aeruginosa isolates from sputum was more than that obtained from other sites. The biofilm formed from environmental isolates was more than that of wound swab isolates but less than that of sputum isolates. There was a difference in the amount of biofilm formed from the isolates. In sputum isolates, 13.33%, 63.33%, and 23.33% produced poor, medium, and high biofilms, respectively. In wound swab isolates, 10% were weak biofilm-formers, 90% were intermediate biofilm-formers, and none of the isolates were high biofilm-formers. In environmental isolates, 30%, 56.66%, and 13.33% were weak, medium, and high biofilm producers, respectively. There was a high biofilm formation in isolates from the sputum and the environment. Biofilm formation in clinical isolates was significantly higher than in environmental isolates.[21]

According to one of the papers released by the National Institutes of Health and the Centre for Disease Control, biofilm-forming bacteria can cause a variety of illnesses and are responsible for 65–80% of infections.[22] Therefore, using biofilm inhibitors is one of the effective methods to

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manage these microorganisms' illnesses. The effectiveness of NPs as biofilm inhibitors against specific bacteria has been demonstrated in several investigations. In one of the research, authors discovered that magnesium oxide nanoparticles had antibiofilm and antiadhesion potentials against drug-resistant bacteria.[23] In the present work, we discussed AgNPs' ability to target *P. aeruginosa* biofilm production. The microtiter plate test was used in the current investigation to measure the percentage inhibition of biofilm development, and the findings showed that there was a 23–86% suppression of biofilm formation in *Pseudomonas aeruginosa* in the presence of AgNP. Our findings are in line with earlier observations by Goswami et al., who investigated how AgNPs affected the formation of biofilms by various bacteria. They discovered that at a concentration of 15 mg/mL, AgNPs may suppress biofilms generated by S. aureus by 89% and those formed by E. coli by 75%.[24] The effectiveness of nanosized silver (at a size of 100 nM) against *P. aeruginosa* was also established by Fattah et al. According to their research, disruption of the extracellular polymeric substances (EPS) matrix resulted in a 95% decrease in the development of biofilm by P. aeruginosa.[25] Our research also provided evidence that the rupture of the EPS matrix may be the source of *Pseudomonas aeruginosa*'s biofilm inhibition. However, NPs can also boost the biomass of the biofilms produced by microorganisms. Contrary to our findings, a prior study by Haney et al. showed that P. aeruginosa biofilm biomass increased after the cells were treated with superparamagnetic iron oxide nanoparticles at a dosage of 0.2 mg/mL. According to their research, cells may employ iron nanoparticles as a source of elemental iron, which would explain why they saw an increase in biofilm biomass and a commensurate rise in cell density.[26]

Conclusion

In this study, different concentrations of hormones were used to produce callus from the leaves of C. colocynthis plant, which was then used in the production of compound green AgNPs. These nanoparticles, when treated with P. aeruginosa bacteria, were able to reduce biofilm formation in all the studied samples.

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Conflict of Interest: None

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