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Procedure Optimization of *Limonia acidissima* Leaf Extraction and Silver Nanoparticle Synthesis for Prominent Antibacterial Activity

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The authors herein report the optimum conditions for the eco-friendly synthesis of silver nanoparticles (AgNPs) using *Limonia acidissima* leaf extract as a reductant. The synthesis of AgNPs using the diluted (0.25 g/mL) leaf extract under direct sunlight produced more homogenous nanoparticles within the shortest possible time. The effects of leaf extraction conditions as well as the reaction time, presence/absence of light, and concentration of the leaf extract as a green reductant for AgNP synthesis are extensively investigated. The synthesized AgNPs have a better homogeneity of size and shape (spherical particles with a size of approximately 24–30 nm and an average size of 27 nm), as confirmed by UV-visible spectroscopy, EDS, and SEM, and the AgNP synthesis was complete within a short time (10 min), as confirmed by colorimetric/optical analysis. The as-obtained nanoparticles show a prominent antimicrobial activity against most of the resistant human pathogens such as *Escherichia coli*, *Citrobacter freundii*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Staphylococcus aureus*.

1. Introduction

The unique properties of silver nanoparticles (AgNPs), such as physical (colorimetric/optical, electrical, and thermal (high electrical conductivity)), chemical, and biological properties, have made them the focus of intensive research in recent times.[1–4] Because of these distinctive properties, AgNPs are widely applied in various fields such as catalysis, optics, antimicrobials, and biomaterial production.[5–10] AgNPs can be synthesized by various approaches such as chemical,[11] photochemical,[12] sonochemical,[13] electrochemical,[14] thermal decomposition,[15] radiation-assisted,[16] microwave-assisted,[17] fungi-mediated,[18] enzyme-mediated,[19] and green chemistry routes.[20–22] Although chemical methods[23–26] can synthesize a large quantity of nanoparticles in a short time, they require reducing agents as well as capping agents for the synthesis and stabilization of the nanoparticles, respectively. Importantly, the chemicals used in the chemical methods for nanoparticle synthesis are costly and toxic and lead to environmentally hazardous by-products. Thus, the search for green, environment-friendly, and time-efficient technologies for the synthesis of not only AgNPs, but also other metal nanoparticles has received increased attention from material scientists.[27]

Green plants usually contain a variety of phytochemicals such as saponins, terpeniods, steroids, tannins, flavonoids, phenolic compounds, phytosterols, and quinones.[28] Some of these phytochemicals act as reducing agents, while some others act as preservatives. Thus, different parts of green plants are used for nanoparticle synthesis. According to literature, different parts and types of green plants such as the leaf of *Saraca indica*,[29] *Lawsonia inermis*,[30] the peel of *Citrus sinensis*,[31] the fruit of *Tribulus terrestris*,[32] the stem latex of *Euphorbia nivulia*,[33] the stem bark of *Callicarpa maingayi*,[34] the root of *Morinda citrifolia*,[35] the leaf of *Limonia acidissima*,[36] and the leaf and bark of *Limonia acidissima*[37] have been successfully utilized for the synthesis of AgNPs.

An investigation of the bio-activity of AgNPs revealed that the size, size distribution, shape, particle composition, particle morphology, particle reactivity in solution, surface chemistry, coating/capping, agglomeration, dissolution rate, efficiency of ion release, and type of reducing agents involved are the determining factors for the bio-activity performance.[38] The physicochemical properties of nanoparticles not only determine the bioavailability of therapeutic agents after both systemic and local administration,[39,40] but also affect the cellular uptake, biological distribution, penetration into biological barriers, and the resultant therapeutic effects.[41,42] Therefore, the development of AgNPs with controlled structures that are uniform in size, morphology, and functionality is essential for various biomedical applications.[43–47] For this purpose, the optimization of synthetic procedure by particular green species is necessary.

Being a medicinal plant, the different parts (e.g., leaf and bark) of *Limonia acidissima* trees can contribute to medicinal treatment. It has been found that both the leaf and bark of

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Limonia acidissima trees contain macromolecules or phytochemicals that are capable of synthesizing AgNPs.[37] Compared with the bark extract, the leaf extract produced larger AgNPs, which consequently exhibited a better antibacterial activity.[37] Based on this phenomena, this study has considered the leaves of *Limonia acidissima* as a potential reductant for the synthesis of AgNPs. It is expected that the optimization of the leaf extraction procedure as well as the AgNP synthesis method will be of great significance for shortening the synthetic time, improving the homogeneity, and enhancing the antibacterial activity. To the best of the authors' knowledge, there is no report on the optimization of *Limonia acidissima* leaf extraction conditions and the synthetic conditions of AgNP prepared using the leaf extract.

In the present study, $AgNO_3$ was used as the precursor and the leaf extract of *Limonia acidissima* trees in an aqueous solution was used as the green reductant. The leaf extract acted not only as a reducing agent, but also as a stabilizing agent. The leaf extract obtained at temperatures were investigated for AgNP synthesis. Moreover, the effects of concentration of leave extract, time, and light on silver nanosynthesis were investigated systematically. The optimized conditions for leaf extraction as well as AgNP synthesis afforded a time-efficient synthesis of AgNPs having better homogeneity, which exhibited prominent antibacterial activity.

2. Results and Discussion

The schematic illustration of the synthetic procedure of AgNPs is shown in Scheme 1.

2.1. Optimization of leaf extraction process

The optimization of *Limonia acidissima* leaf extraction procedure is one of the important steps in green nanotechnologies, as a leaf extract contains many important phytochemicals[28] that are responsible for not only nanoparticle synthesis, but also stabilization. Thus, the extraction process was investigated at both high temperature (60 °C) and room temperature (Figure 1). Although the leaf extract shows similar background absorbances in distilled H_2O at both high temperature (60 °C and 100 °C) and room temperature (Figure 1A), the AgNPs synthesized at room temperature with the leaf extract obtained at room temperature exhibited a higher absorbance (Figure 1B). This is possibly because of some conformational changes in the leaf-extract constituents at high temperatures responsible for AgNP synthesis.

Scheme 1. Schematic illustration of AgNP synthesis from *Limonia acidissima* leaf extract at room temperature.

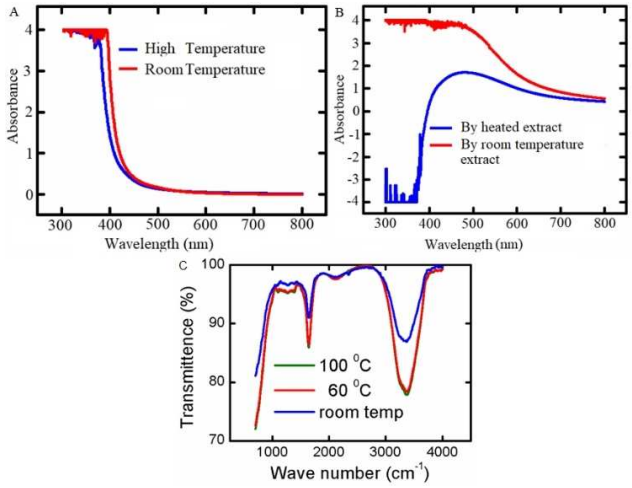

Figure 1. UV-visible spectra of (A) leaf extract at high (60 °C) temperature (blue curve) and room temperature (red curve), and (B) the corresponding AgNP synthesized in 0.0027 M $AgNO_3$ solution with the leaf extract heated at 60 °C (blue curve) and room-temperature leaf extract (red curve) respectively [data for 100 °C is not included]. (C) FTIR spectra of leaf extract at different temperatures.

The FTIR spectra (Figure 1C) of the leaf extract obtained at high temperatures do not show any major changes except in the 1000–1500 cm^{-1} region, and further research is required to confirm the inference. Under these circumstances, room-temperature extraction is considered optimum to obtain a high yield of AgNPs.

2.2. Optimization of AgNP synthetic Procedure

The AgNP synthetic conditions were optimized to devise a simple, cost-effective, and time-efficient method and to improve the homogeneity of the nanoparticles The effects of different parameters such as the concentration of leaf extract, time, and light on AgNP synthesis were investigated (Figure 2). The appearance of the UV absorbance peak at approximately 450 nm (Figure 2A) indicated the formation of AgNPs. Although

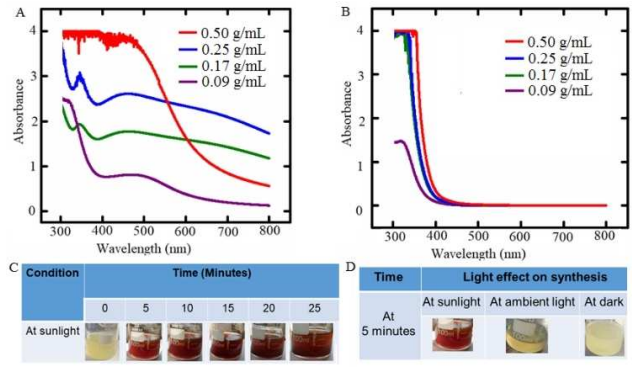

Figure 2. UV-visible spectra of (A) AgNPs synthesized with leaf extract at temperature and (B) leaf extract after different dilutions. Effect of (C) time and (D) light on AgNP synthesis, as determined by colorimetric investigation.

the undiluted leaf extract (0.5 g/mL) exhibited the highest yield which decreased gradually with dilution, a sharp peak appeared for the maximum yield along with a low background response of the leaf extract diluted 2 times (0.25 g/mL) (Figure 2A and 2B). Moreover, the brown solution shown in Figure 2C and Figure 2D confirmed that the nanoparticle formation is not only time-dependent, but also light-dependent. The effect of time and light on AgNP synthesis was investigated in detail and the results are shown in Figure 3. As can be seen in Figure 3A, upon

$$O_2 + (POM)_{red} \xrightarrow{h\upsilon} O_2^{\bullet-} + (POM)_{ox}$$
$$O_2^{\bullet-} + Ag^+ \longrightarrow O_2 + Ag^0$$
$$O_2^{\bullet-} + Ag^0 \longrightarrow O_2 + Ag^{0-}$$
$$Ag^{0-} + Ag^+ \longrightarrow Ag^0 + Ag^0$$

exposure to sunlight, the color changed completely within 10 min of reaction. On the other hand, in ambient light, a brown color was observed at 60 min, whereas in dark, the brown color did not appear. Therefore, it is concluded that a leaf extract concentration of 0.25 g/mL, reaction time of 10 min, and exposure of the reaction chamber to direct sunlight are the optimum AgNP synthetic conditions. The reason for the faster reaction under sunlight than in ambient light is the formation of superoxide free radicals ($O_2^{\bullet-}$) by the phenolic derivatives of the organic matter of the leaf extract in the presence of sunlight, which mediate the formation of AgNPs. This mechanism is supported by literature reports.[48] Figure 3B shows the negligible effect of pH on nanoparticle synthesis in the presence of sunlight as at all pHs, the free radical mechanism is followed.

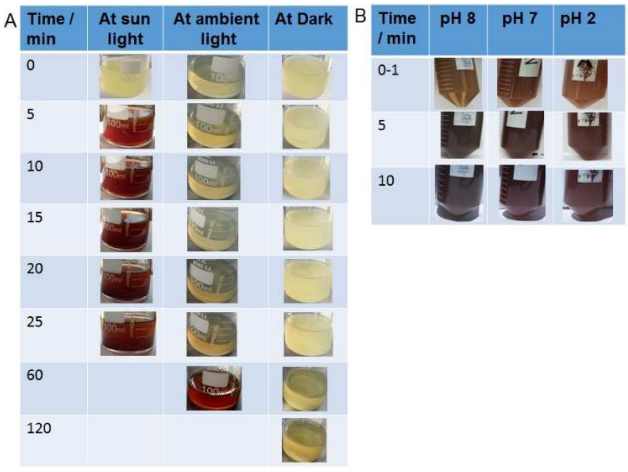

Figure 3. A) Effect of time and light on AgNPs synthesized by the reaction of leaf extract (0.25 g/mL) with 0.0027 M $AgNO_3$ and B) effect of pH under sunlight.

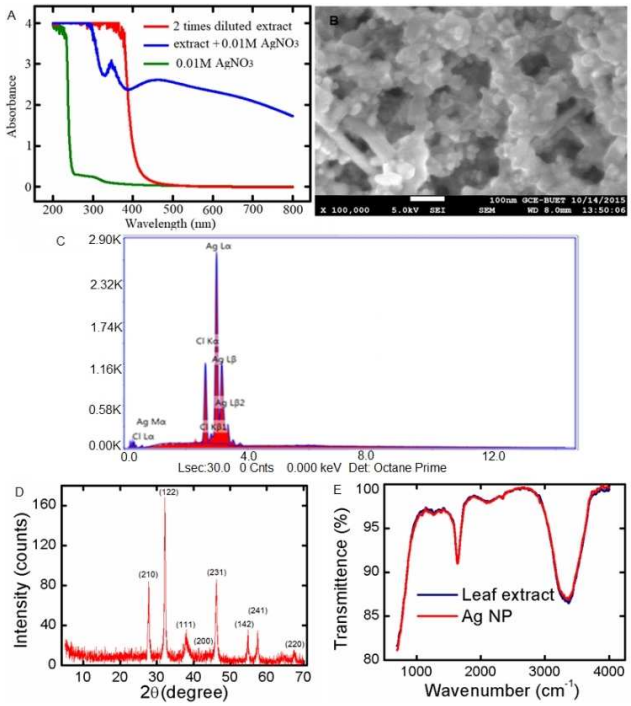

Figure 4. (A) Absorbance spectra of leaf extract (red curve), $AgNO_3$ (green curve), and the mixture of leaf extract and $AgNO_3$ (blue curve), the main reactants involved in the synthesis of AgNP under optimum conditions. The insets show colorimetric images of the respective solutions. (B) SEM image, (C) EDS spectra of the synthesized AgNP, (D) XRD spectra and (E) FTIR spectra of leaf extract and leaf extract mediated synthesis of Ag NP.

2.3. Synthesis of AgNPs at Optimum Conditions and their Characterization

Under the above-mentioned optimum conditions, AgNPs were produced by the time-efficient and homogenous synthesis method, and the UV-visible absorbances of the produced AgNPs

are shown in Figure 4A. As can be seen, the leaf extract and $AgNO_3$ solution do not show any absorbance; however, absorbance can be seen only when AgNPs were formed upon mixing the leaf extract with the $AgNO_3$ solution. The color change of the mixed solution also confirmed AgNP formation. Further, the synthesized AgNPs were characterized by SEM, and the SEM image is shown in Figure 4B. As can be seen, homogeneous spherical AgNPs are formed with a particle size of 24–30 nm. Moreover, the EDS spectrum shows a peak of Ag metal only along with a chloride peak of the solution (Figure 4C), showing the elemental composition. The characteristic Bragg reflections appeared at 2θ angles of 27.78°, 32.18°, 37.92°, 44.8°, 46.16°, 54.76°, 57.52°, and 67.42° corresponding to the (210), (122), (111), (200), (231), (142), (241) and (220) planes of pure Ag with a face-centered cubic structure (JCPDS file no. 04–0783) (Figure 4D). The XRD analysis confirms the crystalline nature of the AgNPs prepared by the plant-mediated synthesis. Moreover, FTIR analysis (Figure 4E) confirmed the

presence of the phytochemicals of the leaf extract on the synthesized AgNPs, which stabilized the nanoparticles.

2.4. Antibacterial Activity of AgNPs

The antibacterial activity of the AgNPs synthesized under the optimum conditions was investigated. Six common bacterial strains, e.g., *Escherichia coli* (Gram negative), *Citrobacter freundii* (Gram negative), *Bacillus subtilis* (Gram positive), *Pseudomonas aeruginosa* (Gram negative), *Salmonella typhimurium* (Gram negative), and *Staphylococcus aureus* (Gram positive), were selected and the activity of AgNPs against these bacterial strains were investigated (Figure 5). The leaf extract was used as a negative control that did not show any activity against any of the bacterial strains; hence, the negative control images were not included here. Ciprofloxacin as a positive control showed activity against the bacterial strains. The results of the antibacterial investigation are summarized in Table 1. Although

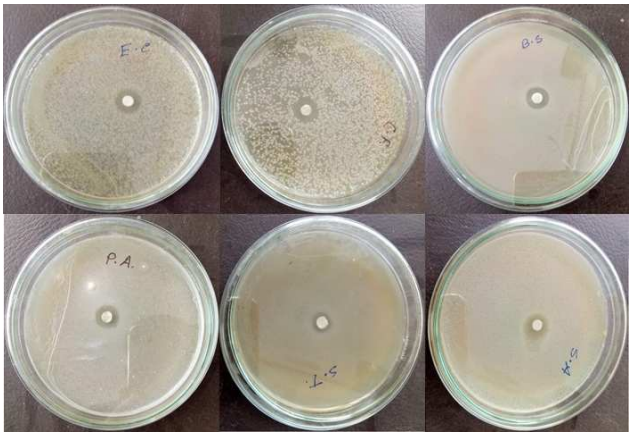

Figure 5. Zone of inhibition images for *Escherichia coli* (E.C.), *Citrobacter freundii* (C.F.), *Bacillus subtilis* (B.S.), *Pseudomonas aeruginosa* (P.A.), *Salmonella typhimurium* (S.T.), and *Staphylococcus aureus* (S.A.).

Table 1. Zone of inhibition is indicated by diameter (in mm) and "-" represents no activity. E.C., *Escherichia coli* (carsgn-2); C.F., *Citrobacter freundii* (JCM-1657); B.S., *Bacillus subtilis* (carsgp-3); P.A., *Pseudomonas aeruginosa* (carsgn-3); S.T., *Salmonella typhimurium* (JCM-1652); and S.A. *Staphylococcus aureus* (cars-2). Ciprofloxacin was used as a standard antibacterial agent. A AgNP dose of 25 µL and standards doses were used, and the concentration was 300 µg mL^{-1}.

Name of bacteria	Zone of inhibition by AgNP (mm)	Zone of inhibition by leaf extract (mm)	Zone of inhibition by Ciprofloxacin (mm)
Escherichia Coli	15	–	45
Citrobacter freundii	16	–	40
Bacillus Subtilis	14	–	35
Pseudomonus aerugenosa	11	–	36
Salmonella Typhimurium	11	–	33
Staphylococcus Aureus	22	–	45

Table 2. Comparison of the antimicrobial activity of the synthesized AgNPs with that reported in literature for *Staphylococcus aureus*.

Size of Ag NP (nm)	Concentration of Ag NP (µg/mL)	Green extract as reductant	Zone of inhibition (mm)	Reference
15	187	*Melissa officinalis* extract	11.5	52
21 to 42	400	*L. acidissima* leaf extract	15.16 ± 1.66	53
32	5000	*Helianthus annuus* leaf extracts	15.5 ± 0.5	54
26	5000	*Ricinus communis* leaf extracts	13.0 ± 0.9	54
27	5000	*Catha edulis* leaf extracts	13.7 ± 0.6	54
8.06 to 91.32	300	*Pantoea ananatis* extracellular extracts	11.30 ± 0.07	55
27	300	*L. acidissima* leaf extract	22	This work

the exact mechanism of the antimicrobial activity of the synthesized AgNPs is difficult to propose except from that reported in literature,[49–51] it is assumed that the AgNPs disrupted the cell membrane (in both Gram-positive and Gram-negative bacteria) and formed reactive oxygen species (ROS) and free radical species such as hydrogen peroxide, superoxide anion, hydroxyl radical, hypochlorous acid, and singlet oxygen within the bacterial cell. The superiority of the synthesized AgNPs was confirmed by the comparison of its antimicrobial activity in terms of the maximum zone of inhibition against Staphylococcus aureus with those previously reported for this bacterial species (Table 2).

3. Conclusions

In conclusion, we proposed the optimum synthetic conditions for AgNPs prepared using the *Limonia acidissima* leaf extract. Room-temperature leaf extraction and a leaf extract concentration of 0.25 g/mL were optimal for AgNP synthesis. Moreover, carrying out the synthetic reaction under direct sunlight yielded AgNPs within the shortest possible time (10 min) along with better homogeneity of size and shape (spherical particles with a size of approximately 24–30 nm and an average size of 27 nm). Most importantly, the as-obtained nanoparticles showed a prominent antimicrobial activity against most of the common resistant human pathogens. Thus, the results of this study will have a significant impact on green technology and biomedical applications. However, a systematic and comprehensive study of the bioreaction mechanism and the downstream pathways is required before we can expect a more meaningful role of homogeneous AgNPs in medicinal applications.

Supporting Information Summary

The complete experimental section.

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Keywords: Antibacterial activity · Green synthesis · *Limonia acidissima* leaf extract · Optimization · Silver nanoparticle

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