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Article

Green Simplistic Biosynthesis of Anti-Bacterial Silver Nanoparticles Using *Annona Squamosa* Leaf Extract

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Abstract

Green nanotechnology involves the synthesis of nanoparticles without the involvement of toxic chemicals. The present study reports the green synthesis of silver nanoparticles from *Annona squamosa* leaf extract rapidly within 20 min. High throughput characterization such as UV-vis spectroscopy, Fourier Transform-Infrared Spectroscopy (FT-IR), Transmission Electron Microscopy (TEM), Field Emission-Scanning Electron Microscopy (FE-SEM), X-ray diffraction (XRD) and Zeta potential measurements disclose the existence of silver nanoparticles. The phenolic compounds present in the aqueous leaves extract paves way for the possible reduction of silver to nano-silver. Electron microscopic studies revealed the average size of nanoparticles were 52 nm. The nanoparticles are highly stable by reaching a zeta potential value of −33.6 mV. Comparative antibacterial efficacy of silver nanoparticles was investigated by disk diffusion and microtitre broth dilution method against *Escherichia coli*. The result showed that silver nanoparticles are toxic to *E. coli* cells at higher concentration. Overall, we suggest microtitre broth dilution method is more reliable to determine the antimicrobial activity of silver nanoparticles than disk diffusion method.

Keywords: Green nanotechnology, *Annona squamosa, E. coli,* disk diffusion, microtitre broth dilution method

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1. Introduction

In recent trends, green nanotechnology is gaining more attention among researchers due to its importance in eliminating the use of toxic chemicals and efficient synthesis of nanomaterials [1]. Notably, metal nanoparticles from bacteria and plants has initiated a new era in the history of nanoparticle synthesis. Moreover, fabricating nanomaterials by "green approach" have less impact on the environment and minimize health associated risks [2]. Significant attempts have been made to synthesis nanostructures with controlled size, shape and properties using biological methods [3]. Conversely, only a few cases report extensive applications in the field of biomedicine and pharmacy [4]. Therefore, designing a precise and powerful nanostructure is mandatory to overcome synthetic materials. Nowadays, nanoparticles of silver, gold, platinum, palladium etc., have been synthesized from plants of natural origin [5]. Among the different nanoparticles synthesized, silver has a remarkable application in the field of sensors, biolabelling, filters, cell electrodes and antimicrobials [6].

Annona squamosa L. is a multipurpose semideciduous small tree and mostly distributed in America and Asia. It shows varied medicinal effects including anti-bacterial, anti-hyperglycemic, anti-genotoxic, anti-hyperlipidemic, anti-oxidant, anti-head lice, anti-diabetic, hepatoprotective, anti-tumour and insecticidal activity [7]. Naturally, *A. squamosa* contain pharmacologically important compounds for potential biomedical applications. In recent years, secondary metabolites belonging to several categories like alkaloids, flavonoids, diterpeniods and phenolic compounds have been studied and published [8]. Hence, in the present study an effort is made to biosynthesis silver nanoparticles rapidly using *A. squamosa* leaf extract. We have also evaluated the antibacterial activity of silver nanoparticles synthesized from *A. squamosa* on *Escherichia coli* by *in-vitro* methods such as disk diffusion and broth dilution method.

2. Materials and methods

2.1 Preparation of aqueous leaf extract

Well matured leaves of *A. squamosa* were collected from Bharathidasan University campus, Tamil Nadu, India. The leaves were surface sterilized with distilled

water, cut into small pieces followed by shade drying for a period of 15 days. The dried materials were grounded into a fine powder by a mixer grinder. A mixture of 200 mg of powdered leaves in 100 ml of distilled water was heated to 60°C for 20 min [9]. The extract obtained was filtered through Whatmann No. 1 filter paper pursued by 0.45 µm sized filters.

2.2 Biosynthesis of silver nanoparticles

For biosynthesis of silver nanoparticles, a blend of 100 µl of 1 mM silver nitrate and 99.99 ml of aqueous leaf broth was heated at 60°C until no further colour change. The colour intensity of colloidal solution gradually increased by the effect of temperature [10]. Precisely after 20 min, there is no change in colour indicates the completion of nanoparticle formation.

2.3 Characterization of silver nanoparticles

The biosynthesized silver nanoparticles were well characterized by high throughput techniques as follows: The optical properties of silver nanoparticles were monitored by a Shimadzu, UV-2450 spectrophotometer at a resolution of 1 nm, between 200-800 nm using 10 mm optical path-length-quartz cuvettes. The molecular functional groups as phyto-constituents present in the silver nanoparticles were determined by Fourier Transform-Infrared studies (FT-IR). FT-IR analysis was carried out by spectrum RX-1 instrument in diffuse reflectance mode operated at a resolution of 4 cm^{-1} of wavelength of about 4000-400 cm^{-1} using KBr pellets. Morphological studies of nanoparticles were done by Transmission Electron Microscope (TEM) and Field Emission-Scanning Electron Microscope (FE-SEM) analysis. TEM images were acquired on a TECHNAI instrument operated at an accelerating voltage of 80 keV. FE-SEM analysis (Carl Zeiss, SIGMA) was done by preparing a thin film of sample onto a copper grid and then allowed to dry using a mercury lamp, prior to measurement. XRD measurements were carried out using a Philps 86 X'Pert Pro X-ray diffractometer by preparing a thin film on glass substrate. Malvern Zetasizer (Nano ZS90, UK) instrument was used to analyse the surface charge (stability) of biosynthesized nanoparticles.

2.4 Antibacterial activity against *E. coli*

The antimicrobial susceptibility of silver nanoparticles against *E. coli* (MTCC 1687) was evaluated by disk diffusion and microtitre broth dilution method as follows:

2.4.1 Disk diffusion method

Disk diffusion assay was adopted to determine the antimicrobial susceptibility of silver nanoparticles [11]. Freshly prepared, Luria bertani (LB) agar plates were swabbed with *E. coli*. The sterile disk (HIMEDIA) of 6 mm was loaded with different volumes of silver nanoparticles (5, 10, 15, 20 and 25 µl). The plates were incubated at 37°C for 24 h for determining the Zone of Inhibition (ZoI).

2.4.2 Microtitre broth dilution method

For microtitre broth dilution [12], sterile 96-well microtitre plates were dispensed with 100 µl of LB broth. Pipette out 100 µl of colloidal nanoparticles solution into the first column wells. By using an 8-channel micropipette mix the content and transfer 100 µl to the rest of wells. Finally discard 100 µl rather dispensing it in column 11. About 5 µl of bacterial suspension was dispensed into all wells of column 1 to 11. The last two wells were used as positive (without antibiotics) and negative controls (left blank without inoculation and antibiotics) respectively. The microtitre plate was closed firmly and kept incubated at 37°C for 24 h. After incubation, the microtitre plates were read at 595 nm using a microtitre reader (BIORAD). Nanomolar concentrations of silver nanoparticles used in the study were prepared by the methodology previously reported by Kumar et al. [13].

3. Result and Discussion

The use of plants in the biosynthesis of metal nanoparticles is a field currently under exploitation. In recent, the extent of silver nanoparticles has drawn the curiosity of many researchers since their biomedical and environmental applications. In the present study, we report on rapid biosynthesis of silver nanoparticle using *A. squamosa* leaf extract. The formation of silver nanoparticles is confirmed by the change in color (pale yellow to brown color) subsequent to the addition of 1 mM silver nitrate with leaf extract [14]. On the other hand, no reaction occurred in control solution (1 mM silver nitrate). The UV-vis absorption spectra of *A. squamosa* mediated biosynthesis of silver nanoparticles from leaf extract are displayed in Fig. 1. It is well known that surface plasmon resonance (SPR) of silver appears between 400 to 500 nm.

Hence, the UV-Vis spectral analysis showed an absorption peak at 435 nm indicating the excitation of SPR [15]. Altogether, the spectral domain of colloidal solution exhibit broader electronic adsorption bands justifying the occurrence of small spherical silver nanoparticles [13]. Comparably, no such peaks were observed in control. We predict, the possible reduction

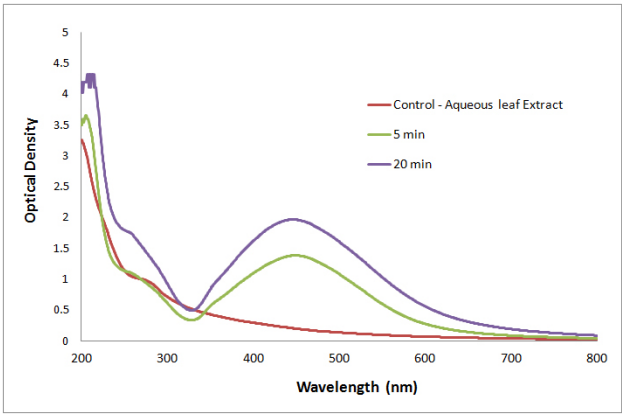

Fig. 1 UV-spectral analysis of silver nanoparticles synthesized from *A. squamosa.*

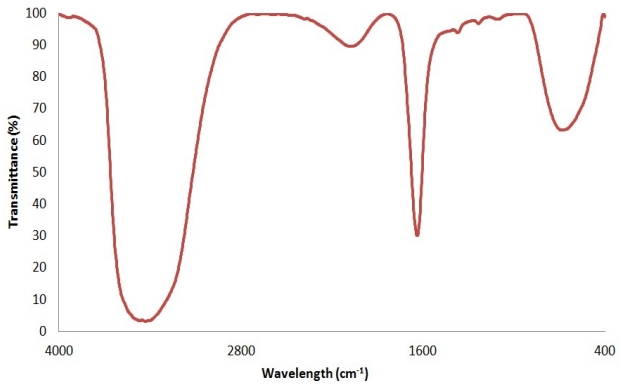

Fig. 2 FT-IR measurement of silver nanoparticles.

of silver ions could be due to the presence of bio-organic molecules in leaf extract. The occurrence of absorption bands between 200 to 230 nm indicates the electronic transition of metallic Ag^{+}. The incidence of major FT-IR spectral signatures at 3429 cm^{-1}, 1637 cm^{-1} and 685 cm^{-1} specify the existence of biomolecules as capping and stabilizing agents for nanoparticles (Fig. 2). A peak at 3429 cm^{-1} designates the pervasiveness of phenolic compounds with free O-H group. The existence of peak at 1637 cm^{-1} and 685 cm^{-1} signify the presence alkenes (C=C) and C-H deformation groups respectively. Based on our results, phenolic compounds play a foremost role in the formation of silver nanoparticles [16].

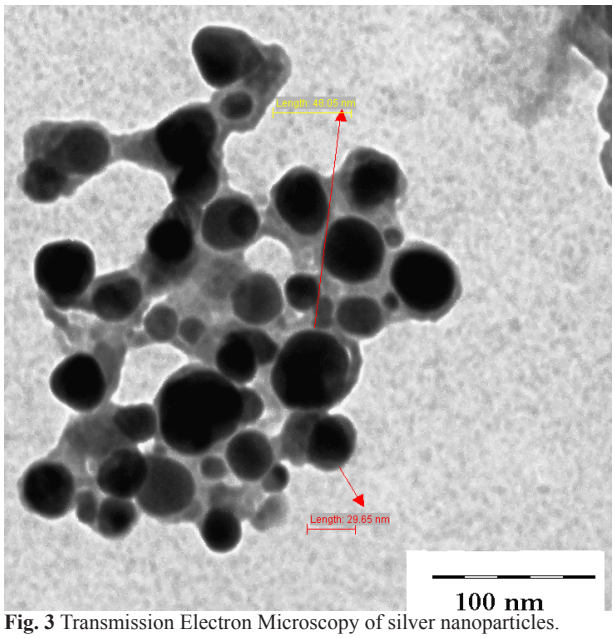

Fig. 3 Transmission Electron Microscopy of silver nanoparticles.

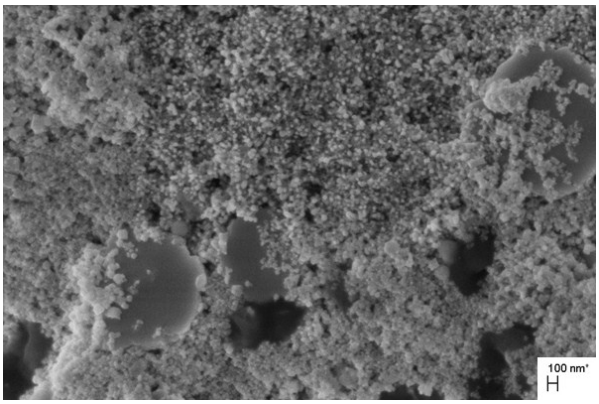

Fig. 4 Field Emission-Scanning Electron microscopic studies

TEM analysis showed the presence of nano-sized spherical shaped metal particles with an average size of 52 nm (Fig. 3). Based on the excitation of SPR, 20 min is well sufficient for the formation of nanoparticles. In accordance with earlier findings [16,17], the formation of nanoparticles is rapid depicting narrow size distribution. The particles display inter-particular distance with no physical contact due to electrostatic repulsive force [3].

Concurrently, the morphological observation made by FE-SEM also revealed the presence of silver nanoparticles with an average particle of size ranged from 20-80 nm (Fig. 4). FE-SEM micrograph suggests, the particles found to be merely agglomerated at higher magnification as compared to TEM image. Previously, it was reported that tannins present in *A. squamosa* may encapsulate silver ions and thereby reducing its aggregation [16]. To summarize these results, the forma-tion of silver nanoparticles is attributed to the presence of reducing agents in the leaf extract of *A. squamosa.*

A typical XRD pattern reveal three intense peaks by 27.74, 32.14 and 46.14 in spectrum of 2θ value ranging from 10-80 (Fig. 5). The Braggs reflection at (1 1 0), (1 1 1) and (2 1 1) were observed corresponding to the formation of silver oxide (Ag_2O). Though, the plausible mechanism involved in the formation of Ag_2O requires additional investigation. We assume, the formation of Ag_2O may perhaps be due to coupling reaction with phenolic compounds. Earlier, we obtain similar kind of results in the biosynthesis of silver nanoparticles using seaweed, *Ulva lactuca* [18]. Altogether, a small peak at 38.14 indicates the formation of pure silver [19]. A few negligible spurious diffraction arises signifying the presence of impurities in the sample. The XRD pattern also reveals that silver nanoparticles formed are crystalline with an estimated size of 52 nm by the Debye-Scherrer's formula, $d = (0.9\lambda \times 180\ °)/\beta\cos\theta\pi$.

Fig. 6 shows the measured zeta potential value of bio-synthesized silver nanoparticles in the colloidal solution. The nanoparticles possess a negative zeta potential value of –33.6 mV and are highly stable due to electrostatic

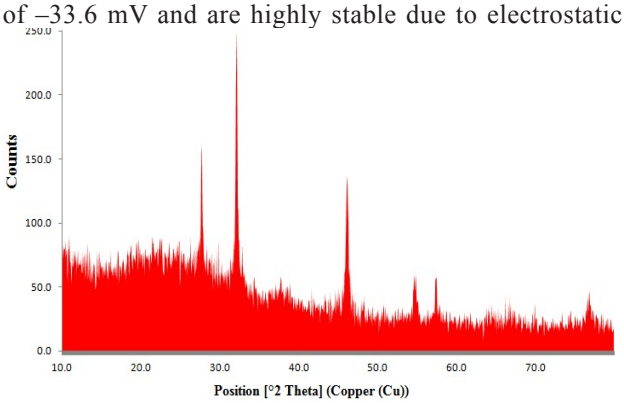

Fig. 5 X-ray diffraction measurement.

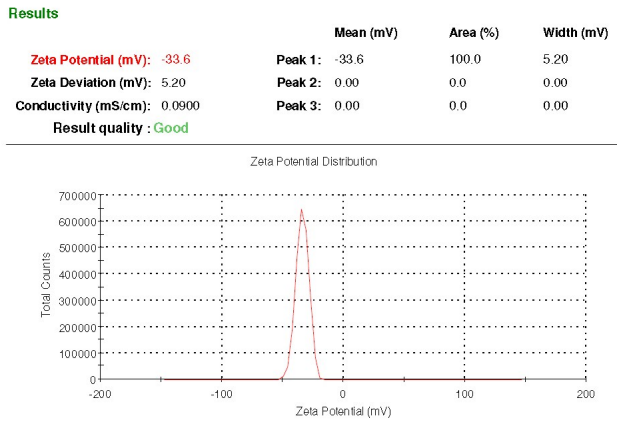

Fig. 6 Zeta potential measurement.

repulsive force. The particles with negative zeta potential strongly interact with cationic additives present in leaf extracts. Earlier report suggests zeta potential value of –37 mV presuming high stability at room temperature were obtained in *A. squamosa* [16]. Interestingly, the metal nanoparticles are stable over a period of month without any special storage in laboratory condition.

According to disk diffusion assay, the antimicrobial effect of silver nanoparticles depends on the ZoI surrounding the sterile disks. This is conceivably because, silver nanoparticles exhibit a wide range of effects which inhibits the growth, loss of infectivity and finally lead to cell death [4]. The above process mainly depends on the susceptibility of microbial species with reference to size [20], shape [21] and concentration of silver nanoparticles [22]. The maximum ZoI by disk diffusion assay based on volume centered at 25 µl (13.1 mm) followed by 20 µl (10 mm), 15 µl (7.2 mm), 10 µl (4.1 mm) and 5 µl (1.9 mm) respectively (Fig. 7). The capping agents present in silver nanoparticles may play a major role in determining the bactericidal effect by enhancing the surface charge density and chemical properties [23]. We also inferred,

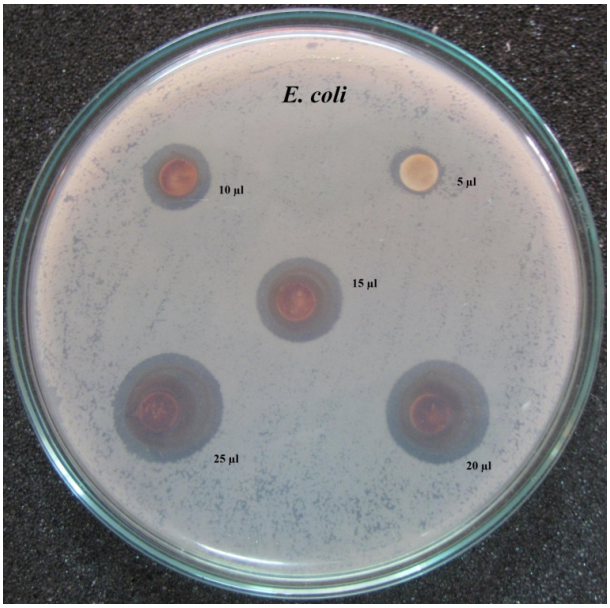

Fig. 7 Disk diffusion assay at different volumes.

E. coli is significantly more susceptible to nanoparticles synthesized from *A. squamosa* leaf extract. However, no inhibition zone occurred in the control (sterile disk) and aqueous leaf broth indicating the absence of antimicrobial activity (data not shown).

For comparative analysis, the antibacterial activities of silver nanoparticles were tested by using the microtitre broth dilution method. The study reveals the minimum inhibitory concentration (MIC_{50}) of silver nanoparticles synthesized by green approach centered at 12.50 nM concentrations (Fig. 8). Further, no promising growth (MIC_{90}) of *E. coli* cells at 100, 50 and 25 nM concentration occurred. As a result, we deem that free radicals from nanoparticles may rupture the cell membrane and depress the activity of membranous enzymes which tend bacteria to eventually die [24]. On the whole, high dosage of silver nanoparticles may perhaps be needed to inactivate or hinder the bacterial growth. Numerous studies have shown the antibacterial effect of silver nanoparticles but still the mechanism involved is unclear and requires further investigation. For the first time, we confer comparative antibacterial effects of silver nanoparticles by disk diffusion and microtitre broth dilution method. The disk diffusion method allows rapid screening of antimicrobials in an efficient and effective manner.

Nevertheless, the measurement is considered to be quantitative describing the susceptibility of antimicrobials based on the ZoI [25,26]. The major downside of this method is unable to develop minimum inhibitory concentration for any bacterial species. Conversely, the microtitre broth dilution method is a qualitative method that proficiently generates minimum inhibitory concentration [12]. Microtitre broth dilution method requires less labour, chemicals and cost effective to screen antimicrobials against numerous bacterial species. It allows rapid screening of microbes by determining the minimum inhibitory concentration compared to other *in vitro* methods. Therefore our study suggests microtitre broth dilution method is more convenient than the disk diffusion method. On the other hand, additional investigation is required to demonstrate the biosafety of silver nanoparticles in animal models before they enter clinical trials.

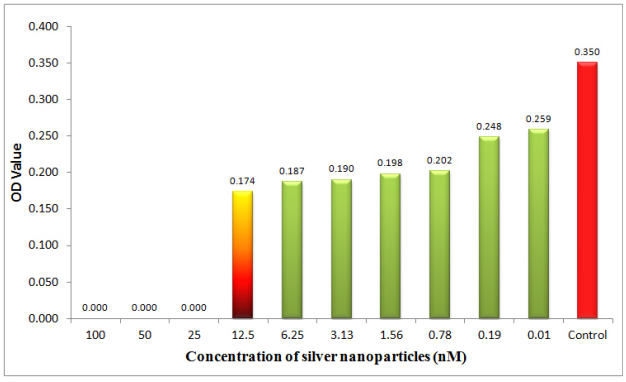

Fig. 8 Microtitre broth dilution method.

4. Conclusion

To conclude, we report on green, rapid, biosynthesis of silver nanoparticles from *A. squamosa* leaves extract. Further, the evidence shown by high throughput techniques like UV-visible spectroscopy, FT-IR, electron microscopic studies, XRD and Zeta potential measurement indicate the occurrence of silver nanoparticles. The antibacterial activity of silver nanoparticles is impressive in hampering the growth of *E. coli*. Our result show, microtitre plate method is appropriate compared to disk diffusion assay by determining minimum inhibitory concentration of silver nanoparticles against *E. coli*. Further, investigations are underway to identify the possible mechanism involved in the bactericidal activity of silver nanoparticles synthesized by *A. squamosa*.

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