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Research Article

Biosynthesis of silver nanoparticles using indigenous *Xanthorrhoea glauca* leaf extract and their antibacterial activity against *Escherichia coli* and *Staphylococcus epidermis*

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ABSTRACT

Background: This study for the first time presents an environmentally friendly, room temperature procedure for synthesizing silver (Ag) nanoparticles via the leaf extract taken from Xanthorrhoea glauca.
Methods: The simple and straightforward green chemistry based technique uses the leaf extract that acts as both reducing agent and capping agent to produce Ag nanoparticles which are subsequently quantified using advanced characterisation techniques. In addition, antibacterial studies were conducted using the Kirby-Bauer sensitivity method.
Results: Advanced characterisation revealed the synthesised particles had a variety of shapes including cubes, truncated triangular and hexagonal plates, and ranged in size from 50 nm up to 200 nm. The Gram-positive bacteria *Staphylococcus epidermis* showed the maximum zone of inhibition at 11 mm.
Conclusions: The study has shown that the leaf extract was able to synthesis Ag nanoparticles with antibacterial activity against *Escherichia coli and Staphylococcus epidermis*.

Keywords: Silver nanoparticles, Antimicrobial activity, Green bio synthesis, Xanthorrhoea glauca

INTRODUCTION

Noble metal nanoparticles such as gold (Au), silver (Ag) and platinum (Pt) have attracted considerable interest in many fields such as medicine, biotechnology, materials science, photonics and electronics.[1-4] The interest in nanoparticles arises from their size, shape and surface morphology that ultimately determines their chemical, physical, optical and electronic properties.[5] Conventional chemical and physical methods used to prepare nanoparticles often use toxic materials and processes that are not environmentally friendly and present many hazards.[6,7] Biological methods for synthesizing nanoparticles offers a green and environmentally friendly technology that can overcome many of the harmful effects produced by conventional chemical and physical methods. Green chemistry methods using biological systems such plants, bacteria, fungus and similar organisms are an attractive and environmentally friendly alternative to chemical and physical methods.[8-12] Plant based synthesis of nanoparticles is normally carried out at moderate temperatures and pH values at atmospheric pressure. The green chemistry based approach also has the advantages of being straightforward processing and eco-friendly. Surveying the literature reveals a wide range of plant extracts have been used to synthesize metallic nanoparticles, in particular Au and Ag have been extensively reported.[13-17] In the case of plants, phytochemicals present in the extracts act as both reducing agent and stabilising agent. Nucleation and

subsequent formation of nanoparticles is dependent on plant species, pH, phytochemical concentration, reaction time and temperature. These factors significantly influence size, morphology and properties of nanoparticles produced over reaction times ranging from a few minutes up to many hours depending on plant species.[18,19]

Historically, the antimicrobial properties of Ag compounds have been used in the medical treatment of infections. The strong antimicrobial activity of Ag against bacteria, fungi and viruses has led to this metal and in particular Ag nanoparticles being extensively studied in recent years.[20] Although the precise mechanisms involved in producing the antimicrobial properties are not fully understood, it is believed the nanoparticles size, well-developed shape and large surface area provides maximum contact with bacteria, fungi and viruses. The particle size, morphological and surface chemistry are believed to be factors influencing the interaction and damage to the cell membrane, and inhibit and damage cellular nucleic acids.[21] Current research has confirmed both antimicrobial and anti-inflammatory properties of Ag nanoparticles. And because of these results Ag nanoparticles have been incorporated into a number of pharmaceutical preparations and wound dressings.[22-23] Future development and incorporation of Ag nanoparticles as antimicrobial agents in pharmaceutical products will require close control of particle size, morphology and particle stability. Furthermore, bio-inspired synthesis of Ag nanoparticles via plants avoids contaminating particles with harmful chemicals and solvents traditionally used in conventional chemical and physical methods.

In the present study, we report for the first time a novel green method for the synthesis of Ag nanoparticles from silver nitrate at room temperature *via* leaf extracts from an Australian indigenous plant Xanthorrhoea glauca as seen in Figure 1c. Biological synthesis was adopted since previous studies by the authors on other Australian indigenous plants have shown that the leaf extracts can act as both reducing agent and stabilizing agent during the synthesis process.[24] Also, the aqueous based technique is straightforward, eco-friendly and produces no toxic waste products.

The Ag nanoparticles synthesized using the leaf extract have been characterized by UV-visible spectroscopy, X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDS), transmission electron microscopy (TEM), field emission scanning electron microscopy (FESEM) and Fourier transform infrared spectroscopy (FT-IR) to determine particle size, morphology, composition and role of the different functional groups in the synthetic process. The antimicrobial properties of the synthesized Ag nanoparticles were evaluated against gram-positive bacteria (*Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli*) using the sensitivity method of Kirby-Bauer.

METHODS

Chemicals

Silver nitrate ($AgNO_3$, (purity: 99.99%)) and sodium borohydride ($NaBH_4$, (purity: 99%) 0 were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and used without further purification. Milli-Q® water was used to make all aqueous solutions used in processing the leaf extracts and chemical synthesis procedures. Milli-Q® water was produced using a Barnstead Ultrapure Water System D11931 (Thermo Scientific Dubuque IA 18.3 MΩ cm-1).

Leaf material and preparation of leaf extract: *Xanthorrhoea glauca* also known as "Grass Tree" leaves were collected from several sites around the Murdoch University campus in Perth, Western Australia. A range of healthy leaves free from damage and ranging from young to mature leaves were harvested from various locations on each plant. The leaves were first washed several times using Milli-Q® water to remove surface contaminants. After washing, 10 g of *Xanthorrhoea* leaves were finely diced and mixed using a standard domestic blender. The leaf mixture was then stirred into a 50 mL solution of Milli-Q® water before being poured into a blending bowl of an IKA® T25 Digital Ultra-Turrax® Homogenizer. The aqueous leaf mixture was homogenized at 5000 rpm for 10 min at room temperature (24°C). After homogenization the mixture was initially filtered using a Hirsch funnel to remove leaf debris. Two further filtrations were carried out using a 0.22 µm Millex® (33 mm Dia.) syringe filter unit. After filtration, the leaf extract was placed into a glass vial ready for nanoparticle synthesis.

Biologically synthesized Ag nanoparticles

Biological synthesis of Ag nanoparticles was examined using varying amounts of leaf extract. Leaf extract solutions consisted of 1 mL for s1, 2 mL for s2 and 3 mL for s3. A 1.0 mL solution of 0.1M AgNO3 was added to each of the 3 leaf extract solutions. Each mixture was vigorously stirred for 1 minute and then allowed to stand at room temperature (24ºC). During which time the colour change of each respective mixture could be easily seen.

Characterization techniques included;

UV-visible spectrum analysis

Analysis was carried out on controls and test samples. Control solutions consisting of Milli-Q® water, $AgNO_3$ solution and filtered pure leaf extracts, (filtered twice, each time using a new Whatman 0.22µm syringe filter). Test solutions consisted of the 3 Ag/leaf colloids s1, s2 and s3. All controls and samples were examined using a Varian Cary 50 series UV-Visible spectrophotometer

(Version 3) over a spectral between 200 and 1100 nm, with a 1 nm resolution at room temperature of 24°C.

Transmission electron microscopy (TEM)

Ag nanoparticle size and morphology was studied using TEM. Sample preparation started with filtering the respective Ag/leaf extract suspensions (filtered twice, each time using a new Whatman 0.22 µm syringe filter). A glass pipette fitted with a rubber bulb was then used to transfer a single drop from each respective sample to its respective carbon-coated copper TEM grid. The grids were then allowed to slowly dry over a 24 hour period. After preparation a bright field TEM study was carried out using a Phillips CM-100 electron microscope (Phillips Corporation Eindhoven, The Netherlands) operating at 80 kV.

X-ray diffraction (XRD) spectroscopy

Crystalline phases present in the samples were identified using XRD spectroscopy. Sample preparation consisted of depositing two to three drops of colloid onto a glass slide via a clean glass pipette fitted with a rubber bulb. The droplets were dispersed over the slide and then dried under vacuum for a period of 4 h. Spectroscopy was performed on each sample in turn at room temperature using a GBC® eMMA X-ray Powder Diffractometer (Cu Kα = 1.5406 Å radiation source). XRD operated at 35 kV and 28 mA in flat plane geometry mode with each scan taking 2 seconds. The respective diffraction patterns were collected over a 2θ range of 20° to 90°.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy was used to identify the major functional groups and vibration modes present in the pure leaf extract and the respective colloids. A Perkin–Elmer Frontier FT-IR spectrometer with Universal Single bounce Diamond ATR attachment was used to analyse samples over a range starting at 525 up to a maximum of 4000 cm^{-1} in steps of 4 cm^{-1}.

Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS): Micrographs showing particle size and morphology were taken using a JCM-6000 (NeoScopeTM) fitted with an energy dispersive X-ray spectroscopy attachment to determine elemental composition of the samples. Individual samples were mounted on substrate holders using carbon adhesive tape before being sputter coated with a 2 nm layer of gold to prevent charge build up using a Cressington 208HR High Resolution Sputter coater.

Antibacterial activity of synthesized Ag nanoparticles: Ag nanoparticle antibacterial activity was studied using two bacterial strains (*Escherichia coli* and *Staphylococcus epidermis*) via the Kirby-Bauer sensitivity method.[25] Bacteria cultures were sub-cultured in Petri dishes (90 mm Dia.) containing a nutrient agar medium. Each strain was uniformly spread over the agar surface in each individual Petri dish using a sterile cotton swab. Then using a micropipette, 15 µL of nanoparticle solution was deposited onto a 6 mm diameter sterile disk (Whatman® AA 2017-006) and then allowed to air dry for 20 minutes. Once dried, the respective disks were placed on the agar media using sterile forceps before the being incubated overnight at 37ºC. After incubation, the diameters of the different zones of inhibition were measured. Sample testing was carried out in duplicate and the mean inhibition zone diameters were used in the subsequent data analysis.

RESULTS

Figure 1a presents a representative UV-Visible spectra for a colloid containing 2.0 mL leaf extract and 1.0 mL of 0.1M $AgNO_3$ (2:1, s2). The spectra displays an optical Surface Plasmon Resonance (SPR) peak at 440 nm, which is typical for metallic Ag nanoparticles. Addition of $AgNO_3$ to the leaf extract changed the colour from clear to a reddish dark brown in 20 minutes. Previous studies report the brown colour indicates the formation of Ag nanoparticles in the reaction mixture due to excitation of surface plasmon vibrations as seen in Figure 1b.[24,26] Mie's theory predicts a single intense SPR peak in the absorption spectra for spherical metal nanoparticles. However, the broad peak centred at 440 nm in this study was due to the anisotropic nature of the nanoparticles. Subsequent TEM analysis revealed particles ranging in size from 50 nm up to 200 nm and a variety of shapes including bars, cubes, truncated triangular and hexagonal plates as seen in the representative image presented in Figure 1d.

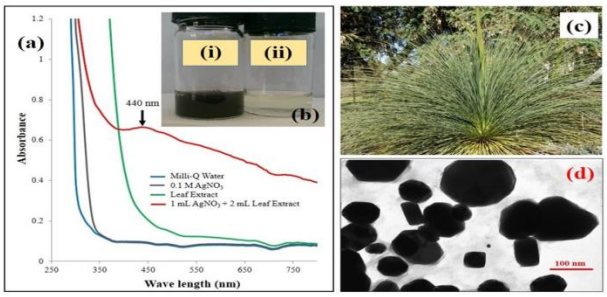

Figure 1: (a) Representative UV-visible spectra for s2 colloid; (b) dark brown colour after reaction (i) and (ii) original clear leaf extract solution; (c) Xanthorrhoea glauca plant specimen used and (d) typical Ag nanoparticles synthesised after 20 minutes duration.

A representative XRD pattern of a dried sample is presented in Figure 2 (a) and reveals three intense peaks corresponding to (111), (200) and (311) Bragg reflections. These peaks indicate an FCC structure present in the synthesized Ag nanoparticles. Also present in the XRD pattern is some degree of peak broadening that indicates the formation of nanoparticles and is

consistent with the results seen in the TEM images. Also present in the XRD patterns were a number of intense peaks (indicated by green stars) associated with unidentifiable materials present in the plant extract. These materials are currently being investigated and will be reported in a future article. Analysis of the respective XRD patterns obtained in this study reveal that they are consistent with similar reports for the biosynthesis of Ag nanoparticles (Figure 2).[27,28]

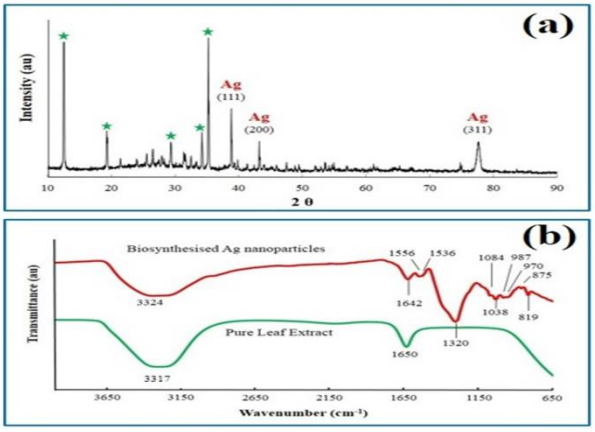

Figure 2: (a) XRD pattern of Ag nanoparticles biologically synthesised using Xanthorrhoea glauca at room temperature and (b) FTIR spectrum of synthesised Ag NPs (red) and pure leaf extract (green).

FTIR analysis was performed on leaf extract samples before and after reaction with $AgNO_3$ to identify potential biomolecules responsible for reduction and capping of the synthesised Ag nanoparticles. The band intensities for different regions of representative spectrums are presented in Figure 2 (b). The broad and intense absorption peak at around 3317 cm^{-1} indicates the presence of O-H functional groups in the pure leaf extract. The shift from 3317 cm^{-1} to 3424 cm^{-1} in the $AgNO_3$ treated leaf extract suggests NH stretching taking place and also indicates the involvement of the O-H functional groups in the synthesis of nanoparticles. Figure 2 (b) also shows the appearance of IR peaks at 1642 cm-1, 1556 cm^{-1} and 1536 cm^{-1} which indicate the presence of amide I and amide II resulting from carbonyl stretching and N-H stretching in the amide linkages in the proteins. Also present in the spectrum is the strong peak located at 1320 cm^{-1} that corresponds to C=C stretching of an aromatic amine.[29-31]

The incidence of carboxyl and amide groups appearing in the $AgNO_3$ treated leaf extract spectrum indicates the involvement of phytochemicals in the biogenic synthesis of Ag nanoparticles.[32] The elemental composition of $AgNO_3$ treated leaf extracts was obtained via EDS analysis to determine the elemental profile of the samples. A representative EDS analysis is presented in Figure 3a and reveals the very strong signal for metallic Ag present in the sample and confirms the results of the XRD study. Figure 3 also contains images of Ag particle growth and presents typical sizes and morphologies present in the samples after 1 h. The dominant shape present at this stage of growth is cubic and the maximum size seen is typically around 1 µm. Hexagonal plates are also seen in smaller numbers and there also a number of truncated triangular plates present. Both triangular and hexagonal plates are also typically around 1 µm in size (Figure 3).

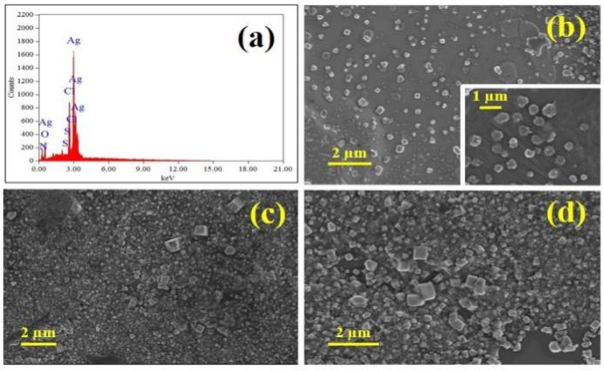

Figure 3: (a) Representative EDS elemental analysis of synthesised Ag NPs; (b)-(d) SEM images of Ag nanometre and micrometre particles.

Antibacterial studies were conducted using the Kirby-Bauer sensitivity method. The method revealed the biologically synthesized Ag nanoparticles displayed significant antibacterial activity against both Escherichia coli and Staphylococcus Epidermis.

Representative results of Ag nanoparticles synthesised from an s2 reaction mixture are presented in Figure 4. The mean diameter of the inhibitory zones were measured and graphically displayed in Figure 4d. The Gram-positive bacteria *Staphylococcus Epidermis* showed the maximum zone of inhibition at 11 mm (Figure 4).

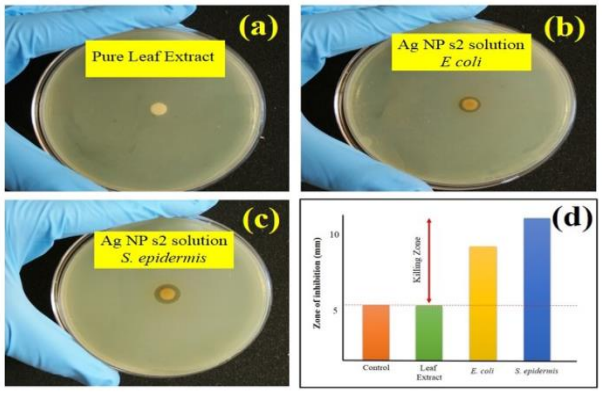

Figure 4: Microbial studies of AG antibacterial effects of (a) pure leaf extract; (b) leaf synthesised AG nanoparticles against e coli, (c) leaf synthesised AG nanoparticles against s epidermis, and (d) zones of inhibition.

DISCUSSION

In the present study Ag nanoparticles were biological synthesized using *Xanthorrhoea glauca leaf* extract. The presence of nanoparticles in the reaction medium was visually indicated via a colour change (clear to dark brown) as seen in Figure 1b insert. UV-Visible spectroscopy revealed a broad SPR peak located at 440 nm, which is similar to peaks reported by Ahmad, N, Sharma using Ananas comosus and by Masurkar et al using cymbopogan citrates (lemon grass) leaf extract.[33,34]

In this study the broad peak was due to the anisotropic nature of the nanoparticles that ranged in size from 50 nm up to 200 nm. But unlike many studies that report spherical morphology, the particles produced by *Xanthorrhoea glauca* leaf extract have a variety of shapes such as bars, cubes, truncated triangular and hexagonal plates as seen in Figure 1d.[33] The anisotropic nature of the nanoparticles synthesised in this study are of particular interest, since studies have shown Ag nanoparticles undergo shape-dependent interactions with cell membranes of micro-organisms.[35]

FTIR studies have revealed that carboxy and amide groups appearing in the $AgNO_3$ treated leaf extract suggest the involvement of phytochemicals in the synthesis of Ag nanoparticles as highlighted by the appearance of IR peaks at 1642 cm^{-1}, 1556 cm^{-1}, 1536 cm^{-1} and 1320 cm^{-1}.[1,36]

The Bragg reflections seen in the XRD patterns were indexed to the (111), (200) and (311) planes of face centred cubic (FCC) orientation and revealed the nanoparticles were pure crystalline Ag. Elemental analysis also confirmed the formation of pure crystalline Ag in the samples. The peak associated with the (111) plane was found to be more intense than the other planes and indicated this plane was the predominant orientation. Also present in the XRD patterns were a number of unknown peaks associated with the leaf extract and are currently being investigated. The presence of these peaks highlights the complex array of phytochemicals present in the leaf extract.

The Ag nanoparticles demonstrated varying degrees of antibacterial activity against *Escherichia coli* and *Staphylococcus Epidermis* as indicated by the inhibition zone diameters. While the leaf extract, like the disk controls didn't display any antibacterial activity as seen in Figure 4.

The Gram-positive bacteria *Staphylococcus epidermis* showed the larger of zone of inhibition (11 mm), compared to Gram negative *Escherichia coli* (8 mm). The variation in inhibition zone size suggests the physical and chemical properties of the cell membranes are disturbed by the attaching Ag nanoparticles. This interaction results in important cellular functions such as permeability and respiration being disrupted. Further damaged is inflicted on bacterial cells by invading Ag nanoparticles interacting with constituents such as DNA and proteins.[35,37]

CONCLUSION

Ag nanoparticles were produced by the interaction of $AgNO_3$ with an aqueous solution containing *Xanthorrhoea glauca* leaf extract. The green chemistry based method is simple and straightforward and was conducted at room temperature. The leaf extract was found to act as both reducing agent and capping agent. Characterisation studies found the synthesised Ag nanoparticles were crystalline, anisotropic, ranged in size from 50 nm up to 200 nm and had a wide range of shapes such as bars, cubes, truncated triangular and hexagonal plates.

The Ag nanoparticles demonstrated varying degrees of antibacterial activity against *Escherichia coli* and *Staphylococcus epidermis*, with *Staphylococcus epidermis* showing a larger zone of inhibition (11 mm). The leaf extract itself showed no antibacterial properties so no synergistic effect was seen in the samples. This green chemistry based procedure is cost-effective, non-toxic and an eco-friendly alternative to conventional physical and chemical processing methods. The procedure used in this study has the potential for large-scale room temperature production of Ag nanoparticles that could be incorporated into antimicrobial based pharmaceutical products.

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