

SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF SILVER AND GOLD NANOPARTICLES PRODUCED USING AQUEOUS SEED EXTRACT OF *PROTORHUS LONGIFOLIA* AS A REDUCING AGENT

R. GANNIMANI^a, A. PERUMAL^a, S. B KRISHNA^a, SERSHEN, K. MUTHUSAMY^a, A. MISHRA^b, P. GOVENDER^{a*}

^a Department of Biochemistry, School of life sciences, University of KwaZulu-Natal, South Africa

^b Department of Applied Chemistry, Faculty of Science, University of Johannesburg, South Africa

The aqueous seed extract of *Protorhus longifolia* was found to be efficient reagent for simultaneous synthesis and functionalization of silver and gold nanoparticles from 1 mM silver nitrate and 1 mM chloroauric acid solution. The progress of the reaction was studied using UV-Visible spectroscopy. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques were used to study surface morphology, size and crystalline nature of nanoparticles. Energy dispersive X-ray analysis confirmed the presence of silver and gold element in the nanoparticle samples. The presence of functional groups over the surface of nanoparticles was confirmed by Fourier transform infrared spectroscopy (FTIR) studies. Zeta potential contributing to the stability of silver and gold nanoparticles was recorded as -21.2 and -19.7 respectively. The modification of the composition and surface morphology of nanoparticles was a result of the capping of organic molecules from the seed extract yielding novel nanoparticles. These nanoparticles showed potential antibacterial activity against bacterial species *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 43300) and *Pseudomonas aeruginosa* (ATCC 27853).

(Received August 21, 2014; Accepted December 10, 2014)

Keywords: *Protorhus longifolia*, Green synthesis, Silver nanoparticles (AgNPs), Gold nanoparticles (AuNPs), Antibacterial activity

1. Introduction

The use of plant extracts for the extracellular synthesis of AgNPs and AuNPs in a bottom-up reduction approach has recently been extensively researched [1]. The interest in these bottom-up methods is based on their ecofriendly nature and less cumbersome reaction procedures. Nanoparticles synthesized using chemical methods have limited application as the toxic chemicals used in the reaction get adsorbed onto the surface of nanoparticles [2]. Intracellular green methods using plants and microbes such as bacteria and fungi generates biocompatible nanoparticles, but at relatively slower rates [3]. On the other hand extracellular methods involving plant extracts are highly convenient due to short reaction times and easy availability of different plant materials as plant organs such as leaves can be harvested without removing the plant from the wild. Plant extracts are often a source of bioactive and medicinally useful compounds and can be generated using a range of plant tissues [4]. In the process of synthesizing nanoparticles from a metal ion solution, medicinally important phytochemicals in the plant extracts reduces the metal ions and also functionalize the surface of the nanoparticles. This functionalization may in this way increase their biocompatibility of green nanoparticles [5]. Steric repulsions between the functionalized molecules can also cause the nanoparticles to form stable colloidal solution and hence use of additional stabilizing chemicals can be avoided [6]. Silver and gold nanoparticles synthesized by

* Corresponding author: govenderpt@ukzn.ac.za

using plant extracts very often have unique chemical and biological properties and are the subject of much interest in the field of catalysis, electronics and most importantly in the medical field as bactericidal and therapeutic agents [7-9]. The chemical composition of plant extract greatly influences on the size and shape of the nanoparticles, which in turn effects on the therapeutic and catalytic properties of nanoparticles[10]. Hence there is necessity to screen more plant extract for the green synthesis of AgNPs and AuNPs of desired size and shape.

There are at present many reports on the extracellular synthesis of silver and gold nanoparticles using various plant parts. Fruit peel of mango [11], lemon [12], Satsuma mandarin [13], were used for the synthesis of AgNPs and AuNPs. Root extract of *Zingiber officinale* [14] was used for the synthesis of AgNPs and AuNPs. Sea weed *Chaetomorpha linum* [15] for the synthesis of AgNPs and flower extracts of *Gnidia glauca* [16] , *Nyctanthes arbortristis* [17], *Carthamus tinctorius L* [18] were used for synthesis AuNPs. Leaf extracts of *Gliricidia sepium* [19] for the synthesis of AgNPs and even seed extracts of *Abelmoschus esculentus* [20] and *Acacia farnesiana* [21] have also been used for the synthesis of AuNPs and AgNPs.

In this present study we investigate the synthesis and antibacterial activity of silver and gold nanoparticles produced using aqueous seed extract of *Protorhus longifolia* (Bernh.) Engl. as a reducing agent. Nanoparticles synthesized using the seeds other species have exhibited bioactivity [22, 23]. *Protorhus longifolia* belongs to family Anacardiaceae is widely distributed throughout South Africa and its bark and leaves are used in traditional medicine to treat hemiplegic paralysis, heart burn and bleeding from stomach [24-27]. The seeds of the plant however, unexplored for their medicinal use. *Protorhus longifolia* produce recalcitrant seeds that are intolerant of desiccation and possibly sensitive to low temperatures, which effectively precludes their storage for any useful period of time [28]. Our interest in using the seeds of these species for the synthesis of nanoparticles is based on the fact that recalcitrant seeds have been shown to possess a number of antimicrobial agents (e.g. β -1,3-glucanase and chitinase) as part of their defense mechanisms against the range of microbes that plague them in the wild [29].

2. Experimental Section

2.1 Materials

Silver nitrate AgNO_3 and Chloroauric acid $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ were analytical grade reagents purchased from Merck Germany. 1% JIK bleach was purchased from Reckitt Benckiser, South Africa. Seeds of *Protorhus longifolia* were collected from Westville campus, University of KwaZulu-Natal (GPS co-ordinates -29.817897, 30.942771).

2.2 Preparation of seed extract

Protorhus longifolia seeds were surface sterilized in 1% JIK bleach, washed with deionized water and then the seeds were air dried at room temperature (28°C) [28]. The dried seeds were ground to fine powder using a commercial blender. Two grams of the fine seed powder was soaked in 25 ml of distilled water and shaken continuously for 24 h. The resulting leachate was filtered and stored at 4°C for further use.

2.3 Synthesis of silver and gold nanoparticles

For the synthesis of nanoparticles the method available in the literature was adopted [30]. 2 ml of plant extract was added to 25 ml of sterile 1 mM silver nitrate solution and 1 mM chloroauric acid solution and incubated with continuous stirring on the rotary shaker at 120 rpm, 30°C for 24h. Progress in the reaction was monitored by measuring the UV absorbance.

2.4 UV-Vis Spectrum analysis

The reduction reaction of silver and gold ions by aqueous seed extraction was followed by UV visible absorption measurements at room temperature as function of time using Specord 210, Analytikjena spectrometer. The broad absorbance peaks between 400-500 nm and 500-600 nm confirmed the formation silver and gold nanoparticles respectively. 1 mM AgNO₃ and 1 mM HAuCl₄ solution were used as the blank solution for these measurements [31].

2.5 Transform Infrared (FTIR-ATR) Spectroscopy Measurements

The nanoparticle colloidal solution was centrifuged and washed with distilled water to remove any unreacted materials. AgNPs centrifuged at 4000 rpm for 20 min, whereas AuNPs centrifuged at 8000 rpm for 20 min. The nanoparticle pellet thus obtained was dried in a desiccators containing calcium chloride and thereafter used for the FTIR studies using Perkin Elmer Precisely Spectrometer 100 FTIR-ATR to identify the functional groups present on the surface of nanoparticles.

2.6 Scanning Electron Microscopy Measurements

Morphological studies of the synthesized AgNPs and AuNPs were carried out using scanning electron microscopy (FEGSEM ZEISS ULTRAPLUS). Samples prepared by adding a drop of nanoparticle solution over the carbon tape glued over metal stub. The samples thus obtained were coated with carbon and observed at 10000 X magnification. The nanoparticle pellet obtained after purification was subjected to energy dispersive X-ray analysis (SEM-EDX) at 20 kV using AZTEC software for the analysis.

2.7 Transmission Electron Microscopy (TEM) Measurements

Both silver and gold nanoparticle solutions obtained were sonicated for 15 min to disrupt any possible aggregates [32]. A small amount of this sonicated suspension was placed on carbon coated copper grid and dried under infrared light for solvent evaporation. High-resolution TEM images were obtained on JEOL TEM model no 2100 operating at accelerating voltage of 200 kV and 0.23 nm resolution. ImageJ software was used to analyze the nanoparticle size distribution and resultant data were plotted in histograms.

2.8 Zeta potential measurements

The Zeta potential of green synthesized AgNPs and AuNPs was determined at 25°C using Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). Water was used as dispersant. Measurements were carried out in triplicates [33].

2.9 Antibacterial activity

Minimum Inhibitory Concentration

MIC, defined as the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism after overnight incubation, was determined by monitoring the growth of bacteria in a microplate reader (Synergy HT, BioTek Instruments) at 630 nm by micro dilution method as per NCCLS guidelines [34]. The bacterial test cultures used in this study were *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 43300), *Enterococcus faecalis* (ATCC 5129) and *Pseudomonas aeruginosa* (ATCC 27853). Serial twofold dilutions of AgNPs and AuNPs solutions were prepared in sterile 96-well plates over the range of 200-1.25 µg/ml [32]. The wells were then inoculated with diluted overnight broth culture initially adjusted to 0.5 McFarland turbidity standards and incubated at 35°C for 24 hours. Neomycin (Sigma) served as a positive control; MIC was recorded as the

lowest concentration at which no growth was observed. All the experiments were carried out in triplets.

3. Results and discussions

3.1 UV -Visible studies

Surface plasmon resonance a unique optical phenomenon of metal nanoparticles, in the UV region enables their easy detection. This property arises due to surface plasmon oscillation of free electrons [35]. Nanoparticles show strong absorbance in the UV region due to this phenomenon and the wavelength at which absorbance occurs is a characteristic property of particular metal nanoparticle and also depends on the size and shape of particles [36]. **Fig.1** illustrates the UV-visible absorbance spectra of reaction mixture containing aqueous seed extract with silver nitrate (1 mM) and chloroauric acid (1 mM) at various time intervals. After the addition of the seed extract, the silver nitrate solution changed to brown and gold chloride solution changed to wine red. The intensity of the color and absorbance of both the solutions increased with the time as the reaction proceeded.

Both the AgNPs and AuNPs reaction mixtures showed a single broad surface plasmon resonance band between the wavelength 400-500 nm and 500-600 nm respectively. The change of color of reaction mixture within 15 min indicated that reduction of Ag^+ and Au^{3+} is a rapid reaction. The reactions were carried out for 24 h to make sure they run to completion.

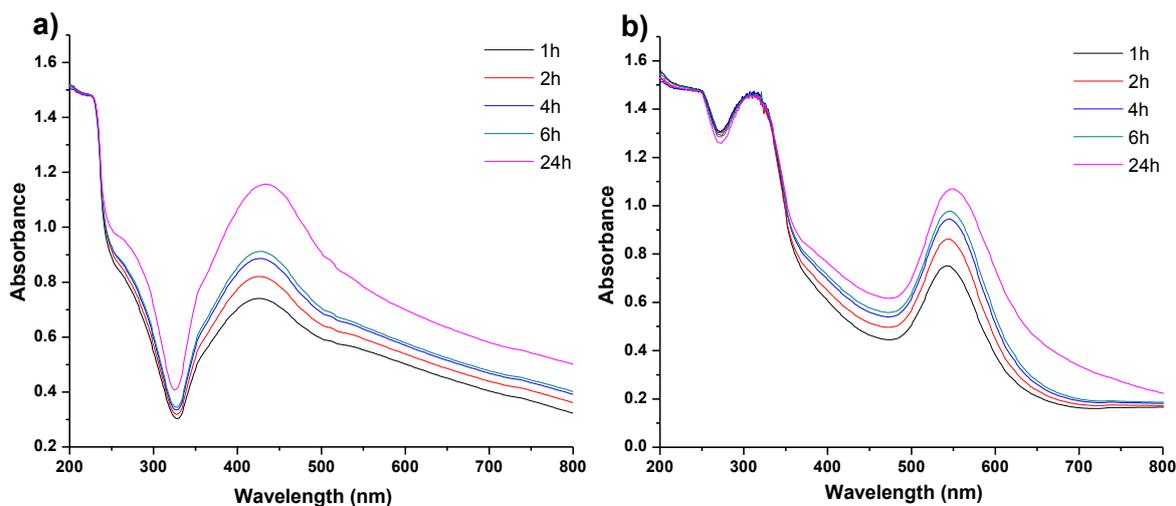


Fig.1 UV-Vis spectra recorded as a function of reaction time a) AgNPs and b) AuNPs

3.2 FTIR-ATR studies

The IR spectrum results showed that some organic biomolecules from seed extract had become associated with the surface of silver and gold nanoparticles to form a capping agent. The steric repulsion between the capped molecules contributes to the stability of nanoparticles. The major IR bands at 3258, 1629, 1514, 1055 cm^{-1} for AgNPs (**Fig.2a**) and the IR bands at 3263, 1739, 1635, 1530 and 1160 cm^{-1} for AuNPs (**Fig.2b**) were observed. The broad spectrum of IR peak at 3260 cm^{-1} for both AgNPs and AuNPs referred to the strong stretching vibrations of -OH functional group. The band at 1160 cm^{-1} and 1055 cm^{-1} can be assigned to the ether linkages or -C-O- functional group. To a large extent, these bands might be the product of -C-O- groups of the polyols such as flavones, terpenoids and the polysaccharides of seed extract. The absorbance band centered at 1635 cm^{-1} and 1629 cm^{-1} is associated with the stretching vibrations of -C=C- or aromatic groups. The band around 1739 cm^{-1} for AuNPs can be assigned to C=O stretching

vibrations of the carbonyl functional group in ketones, aldehydes, and carboxylic acids. The spectrum also exhibits two intense bands at 2851 cm^{-1} and 2919 cm^{-1} , for AuNPs which is assigned to the symmetric and asymmetric stretching vibration of sp^3 hybridized $-CH$ groups.

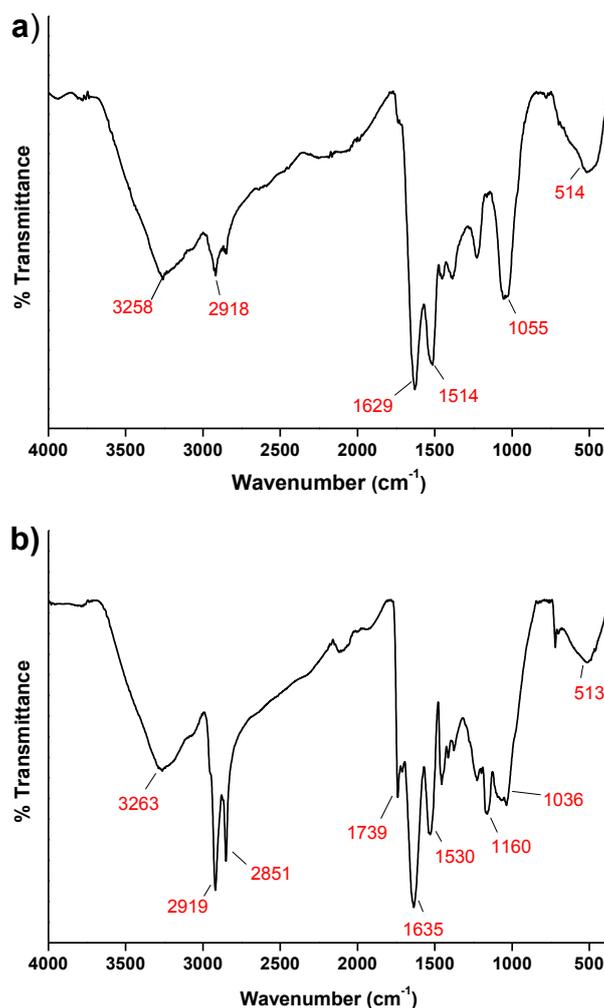


Fig.2 FTIR ATR spectra of a) AgNPs and b) AuNPs

3.3 SEM and TEM Analysis

SEM analysis confirmed that particles are of a nano-size. The spherical shape of the nanoparticles is shown in the SEM images (Fig.3a and 3b). The SEM-EDX analysis confirmed that the particles were composed of elemental silver and gold (Fig.3c and 3d).

TEM analysis showed that both silver and gold nanoparticles were poly-dispersed and predominantly spherical or polyhedral in shape. The majority of the silver nanoparticles were 20-30 nm in size whereas AuNPs were in the range of 10-20 nm. Fig.4A, a and Fig.4B, a shows clear morphology of silver and gold nanoparticles. The Selected area diffraction pattern clearly indicated that the AgNPs and AuNPs formed by the reduction of metal ions by the seed extract are polycrystalline in nature with clear lattice fringes (Fig.4A, b and Fig.4B, b) characteristic of crystalline nature of nanoparticles [37, 38].

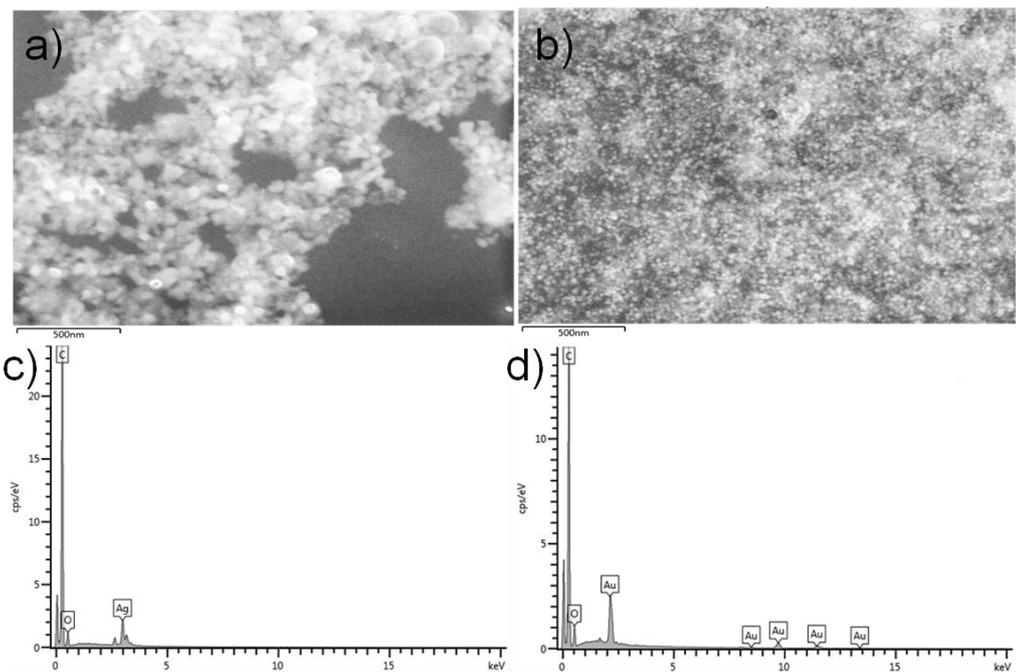


Fig.3 SEM morphology a)AgNPs and b) AuNPs and SEM-EDX pattern c) AgNPs and d) AuNPs

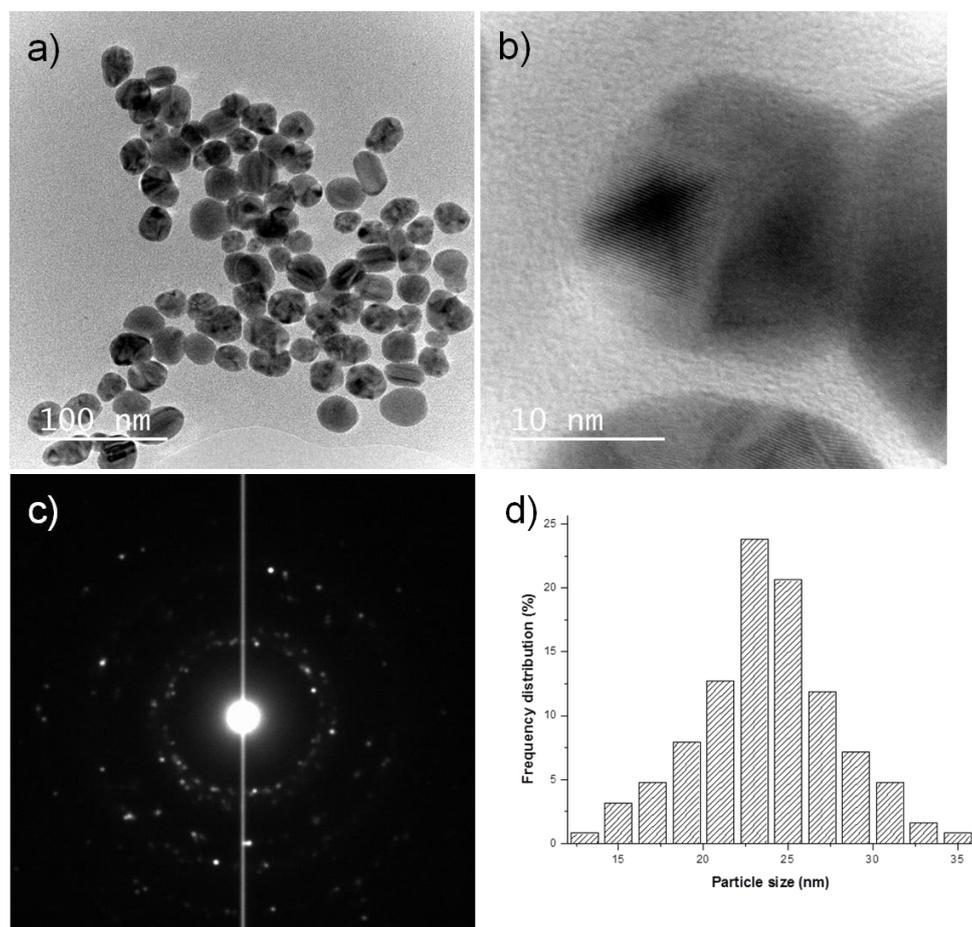


Fig.4A a) HRTEM micrograph b) Lattice fringes c) SAED pattern and d) Histogram of AgNPs

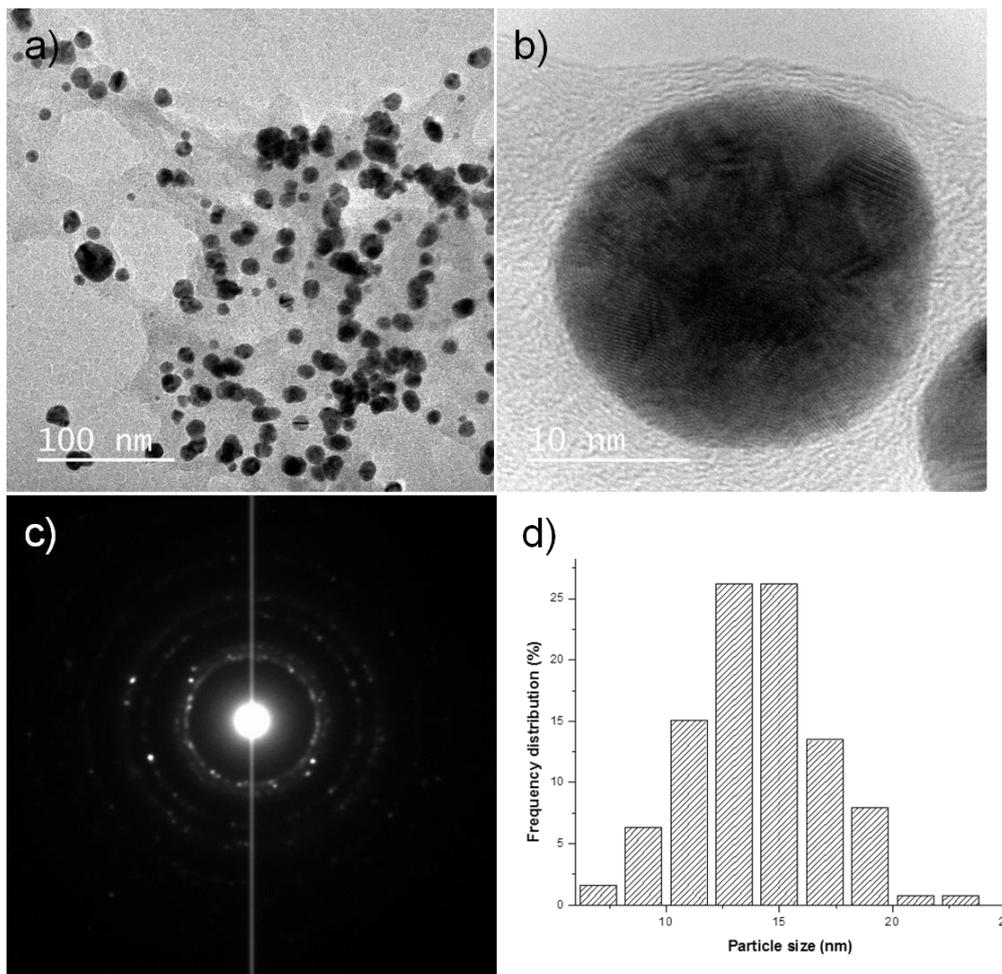


Fig.4B a) HRTEM micrograph b) Lattice fringes c) SAED pattern and d) Histogram of AuNPs

3.4 Zeta potential measurements

The electric charge on the surface of nanoparticles can be measured in terms of Zeta potential. Zeta potential was found to be -21.2 mV for AgNPs and -19.5 mV for AuNPs. The negative zeta potential confirms the negative charge on the surface of colloidal nanoparticles. The coulombic repulsion forces induced by surface negative charge minimize the aggregation and thus contribute to the stability of the green synthesized nanoparticles [33].

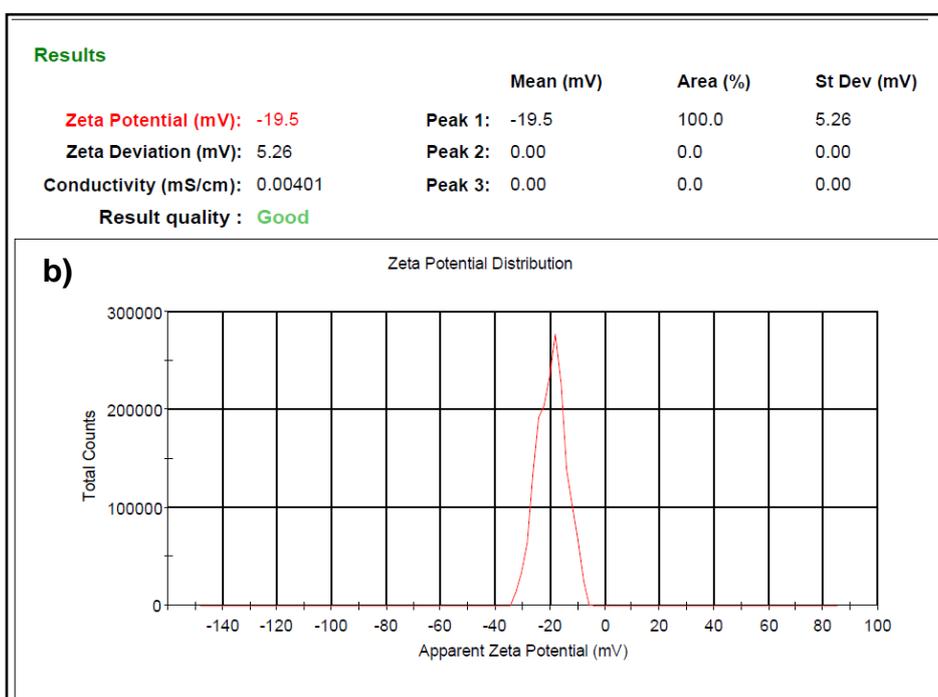
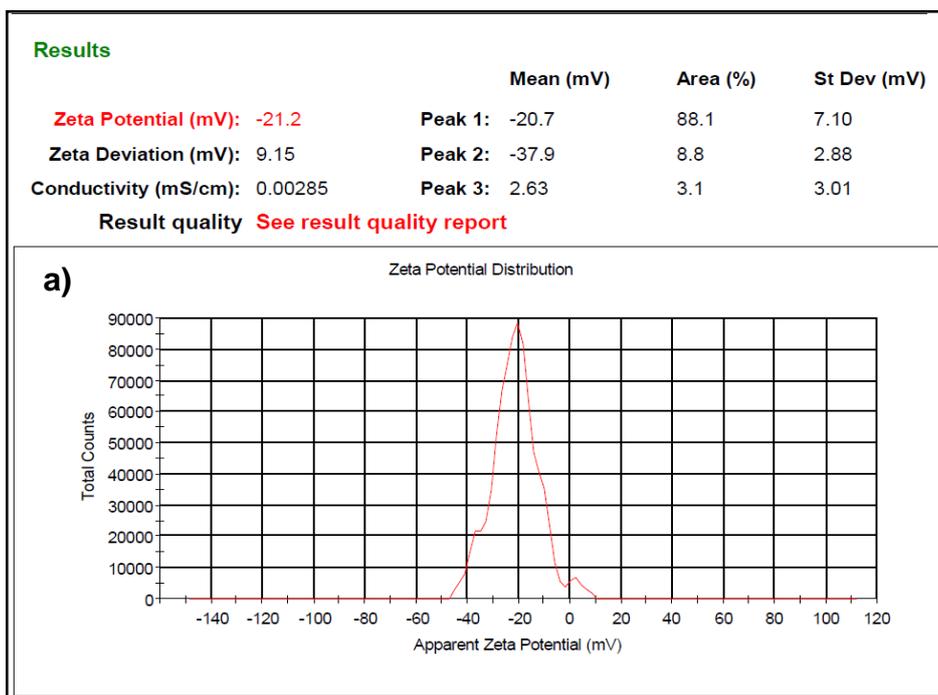


Fig.5 Zeta potential graphs of a) AgNPs and b) AuNPs

3.5 Antibacterial Studies

The use of nanoparticles functionalized with antibacterial compounds is an interesting strategy to overcome the problem of multidrug resistance by bacteria [39]. Inorganic metal nanoparticles have been shown to target multiple components of cell and hence, there is less scope for bacteria to develop resistance [40]. The antibacterial mechanisms of action of metal nanoparticle include cell wall disruption followed by leakage of cell content, binding of metal ions to DNA and proteins which results in inactivation or severe alteration of cellular metabolism [41]. Further properties of nanoparticles such as high surface area, slow and steady release of metal atoms compared to salt or bulk metal add to their antimicrobial properties.

In the present study silver and gold nanoparticles showed good antibacterial activity against tested pathogens (**Table 1**). These results also showed that AgNPs had higher antibacterial activity than AuNPs. This is in accord with other studies which have shown AgNPs to be more antibacterial than AuNPs, largely due to the relatively inert chemical nature of gold [40]. *E. coli* was the most sensitive to silver nanoparticles (MIC 3.12 µg/ml) followed by *P. aeruginosa* and *S. aureus* (MIC 50 µg/mL). These results are in agreement with other studies [42]. *E. faecalis* was resistant to silver and gold nanoparticles. Similar results were observed in a previous study where silver nanoparticles were more active against gram negative bacteria than gram positive bacteria and this was attributed to change in the cell wall composition of bacteria [43]. Gold nanoparticles were effective at 200 µg/ml amongst the varied concentration range against the tested pathogens. The seed chemical content adsorbed onto the surface of nanoparticles could have added to their antibacterial activities and thus nanoparticles may have acted as carriers or drug delivery systems for the antibacterial content of plant material functionalized on their surface [32]. Recent reports also suggest similar mechanism where gold nanoparticles functionalized with small molecules have shown good antibacterial activity [44].

Table 1. Minimum inhibitory concentration of *Protorhus longifolia* seed extract derived silver and gold nanoparticles against bacteria

Compound	Minimum Inhibitory Concentration (µg/ml)				
	Gram negative			Gram positive	
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. faecalis</i>
AgNPs	3.12	100	50	50	NA
AuNPs	200	200	200	200	NA
Neomycin	5	10	5	5	20

MIC was determined by microbroth dilution technique and values reported in the table represent the values obtained in triplicate. Data reported as the mean±standard error of the mean ≤5.

4. Conclusion

In conclusion, we successfully synthesized silver and gold nanoparticles using aqueous seed extract of recalcitrant seeded *Protorhus longifolia* and confirmed the antibacterial activity of resultant nanoparticles. The reduction reaction for the synthesis of nanoparticles was rapid under the ambient conditions and reaction handling was easy since it does not require boiling or subsequent treatment. The nanoparticles produced showed distinct poly-dispersity. The IR studies confirmed the capping of organic contents of seed extract of *Protorhus longifolia* onto the surface of nanoparticles. The capping of nanoparticles by organic molecules found in the seed extract and the negative charge of nanoparticles is suggested to have added to their stability. These findings would significantly contribute to the advancement in the formulation of novel phytochemical based antimicrobial agents.

Acknowledgement

This research was funded by National Research Foundation (NRF) of South Africa, the University of KwaZulu-Natal, South Africa.

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