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Green synthesis of silver and gold nanoparticles using fruit extract of *Sapindus mukorossi'* and their antibacterial activity.

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ABSTRACT

Silver and gold nanoparticles are synthesized through a facile and green method using aqueous extract of important natural resource, Sapindus mukorossi which is commonly known as soap nut. In this present synthesis soap nut extract are being used as both reducing as well as stabilizing agents at ambient condition. Silver (AgNPs) and gold (AuNPs) metal nanoparticles are characterized by HRTEM, UV-vis, EDX measurements. The peaks of UV-VIS absorption spectra suggest the formation of silver and gold nanoparticles. The different shape and size of nanoparticles are confirmed by transmitted electron microscopy (HRTEM). Synthesized silver and gold nanoparticles are spherical in shape with diameter ~ 2-5 nm and ~ 10-20 nm respectively. XRD study shows that the particles are crystalline in nature with face centered cubic geometry. Apart from all aspects this it is a pollution less eco-friendly synthetic process. Antibacterial activities of the synthesized nanoparticles are investigated against two Gram-negative (Escherichia coli and Pseudomonas aeruginosa) and two Gram-positive (Staphylococcus aureus and Bacillus thuringiensis) bacteria using the disc diffusion method. AgNPs show inhibition activity against Escherichia coli nearly equivalent to the commercially available antibacterial drug e.g. Ampiciline. Our observed minimum inhibition concentration (MIC) for AgNPs and AuNPs which inhibit 10⁶ cfu/mL E. Coli in liquid Mueller-Hinton medium are 32 µg/mL and 40 µg/mL.

KEY WORDS: *Sapindus mukorossi*, Silver nanoparticles, Gold nanoparticles, Antibacterial activity, MIC, Green synthesis.

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INTRODUCTION

Area of nanoparticle research has witnessed tremendous growth due to unusual chemical and physical properties demonstrated by this intermediate state of matter.¹ During the last two decades, research on inorganic nanoparticles had been developed rapidly due to their exceptional physical and chemical properties that are quite different from the bulk one.² Among the noble metal nanoparticles silver and gold are the most widely recognized for their unique size dependent optical and electronic properties³ with potential applications in the various field of micro-electronics,⁴ photocatalysis,^{5,6} lithography,^{7,8} electron microscopy marker,⁹ DNA sequencing,¹⁰ medical sciences^{11,12} etc.

Nanotechnology has attracted a lot of attention because of our increasing ability to synthesize and manipulate the properties of nanoparticles according our demands. Various techniques, including chemical and physical means are developed to prepare the metal nanoparticles, such as chemical reduction,^{13,14} electrochemical reduction,^{15,16} photochemical reduction^{17,18} and so on. The above methods use various toxic organic surfactants or solvents as surface passivators to prevent the particles from agglomeration. These organic surface passivators cause pollution and environmental hazards. Now there is a growing need to use nontoxic, environmentally benign, renewable materials for the synthesis of nanoparticles. So intentions of researchers have turned towards green synthesis. The metal-microbe have important role in several biotechnological applications and microorganisms are considered as potential bio-factory for synthesis of nanoparticles.¹⁹⁻²³ Use of plant extract for the synthesis of nanoparticles could be advantageous over other environmentally benign biological processes by eliminating the elaborate process of maintaining cell cultures. The formation gold and silver nanoparticles by living plants were first reported previously.^{24,25} The above synthetic protocol by plant extract or biomass exemplifies the promising application of the green synthesis of metal nanoparticles. Very recently silver and gold nanoparticles have been synthesized using various natural products like green tea (*Camellia sinensis*),²⁶ neem (*Azadirachta indica*) leaf broth,²⁷ latex and seed extract of Jatropha curcas,^{28,29} aloevera plant extract,³⁰ leaf extract of Hibiscus rosa sinensis,³¹ Rosa rugosa,³² black tea leaf extract,³³ leaf extract of Cocculus hirsutus,³⁴ mushroom extract,³⁵ tansy fruit extract³⁶ etc.

It is well known that silver and its compounds have effective antimicrobial activities since the ancient age. The continuous search for potential antimicrobial agents revealed that silver nanoparticles have broad spectrum of antimicrobial activities for bacteria, fungi and virus³⁷⁻⁴⁰ investigated the antibacterial activities of hydrophilic silver nanoparticles against two Gram positive and three Gram negative bacteria and their obtained MIC values showed that the phase transferred silver nanoparticles were the promising antibacterial agents. Li et al. proposed the plausible

mechanism of antibacterial action of silver nanoparticles on *Escherichia coli*⁴¹. They suggested that the structure of bacterial cell membrane are damaged and depressed the activities of membranous enzyme causing the death of bacteria. The variation in antibacterial activities of silver nanoparticles with the size of particles was reported by Martinez-Castanon et al.⁴² and they suggested that the size is an important factor to inhibit the bacterial growth. Smetana et al.⁴³ represented biocidal activities against *Escherichia coli* and *Staphylococcus aureus* of nanocrystalline silver powder and observed that very small, irregular surfaces are necessary for high biocidal activity.

In this present investigation, we report a green method for the synthesis of silver as well as gold nanoparticles using soap nut (*Sapindus mukorossi*) aqueous extract and no toxic chemicals are used as reducing or stabilizing agent during the synthesis. Fruits of *Sapindus mukorossi* (soap nut), commonly known as Ritha or Aritha are found throughout India. Pericarp of this fruit forms soapy lather in water and is used for washing garments and also for other cleaning purposes. Major active constituents of fruit of *Sapindus mukorossi* are saponins (11.5%), sugars (10%) and mucilage.⁴⁴ The saponins are glycosides with foaming characteristics. It consists of polycyclic aglycones attached to one part which is called sapogenin. The foaming ability of saponins is caused by the combination of hydrophobic (fat soluble) and a hydrophilic (water soluble) sugar part. Four oleanane type saponins and two new dammarane type saponins along with seven known saponins are isolated from *Sapindus mukorossi*.⁴⁵ The poly hydroxyl sugar moiety of saponins binds the silver nanoparticles and thus prevents it from agglomeration. The non sugar part of saponins has a direct antioxidant activity and it helps both the silver and gold nanoparticles to survive for long time in open atmosphere. This huge source of natural surfactants draws our attention and motivates us for the present synthesis of silver and gold nanoparticles.

We have tested antibacterial activities of the silver as well as gold nanoparticles against four pathogenic bacteria *E. coli*, *P. aeruginosa*, *S. aureus* and *B. thuringiensis*. The silver and gold nanoparticles that synthesized by present green route are found highly toxic against human pathogenic bacteria and formed clear inhibition zone which are comparable to the standard antibiotics like Ampicillin, Rifampicin and Norfloxacin. The minimum inhibition concentration (MIC) results indicate that 32 µg/mL silver nanoparticles and 40 µg/ml gold nanoparticles inhibit the growth of 10^6 cfu/mL *E. coli* cells in liquid Mueller-Hinton medium.

MATERIALS AND METHODS

Materials:

The fruits of *Sapindus Mukorossi* (soap nut) were collected from local sources. Silver nitrate (AgNO₃) and Chloroauric acid (HAuCl₄, 3H₂O) of analytic grade were purchased from Sigma-Aldrich. All aqueous solutions were prepared using triple distilled deionized water.

Organism, medium and cultivation:

The strain of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Bacillus thuringiensis* (ATCC 12228) were purchased from American Type Culture Collection (ATCC). Mueller-Hinton medium was purchased from HiMedia Laboratories Pvt. Ltd (India). Inhibition zone formation was studied out by disc dispersion method. Minimum inhibition concentration (MIC) of microorganism and the growth curves for *E. Coli* exposed and unexposed to silver nanoparticles were determined using the value of optical density (OD) at 610 nm.

Preparation of Extract:

Fruits of '*Sapindus mukorossi*' were washed thrice with distilled water and dried in vacuum for 24 hours. The pericarps of the soap nut were separated from seed and finally cut into small pieces. One gram of it was boiled with 100 mL distilled water for 1 hour. Pale yellowish coloured extract was collected after filtration.

Synthesis of Ag and Au nanoparticles:

20 mL aliquot of soap nut extract was mixed with 20mL aqueous solution of 0.1 mM silver nitrate. Mixture was heated at 80°C with constant stirring for 1 hour in oil bath. Appearance of golden yellow colour indicates the formation of silver nanoparticles. Golden yellow colour became intense with the increasing concentration of AgNO₃ solution from 0.1 mM to 100 mM. In a similar reaction 20mL soap nut extract was mixed with 20 mL 5 mM aqueous HAuCl₄ solution and the resulting solution was heated at 80°C. Appearance of violet colour indicates the formation of AuNPs. Intensity of violet colour gold sol deepen with the increasing concentration of HAuCl₄ solution from 5 mM to 50 mM.

Characterization of the silver and gold nanoparticles:

UV-vis spectroscopic study was carried out using Shimadzu UV-1601 spectrophotometer. Morphology and size of metal nanoparticles were characterized by JEOL-JEM-2100 higher resolution transmission electron microscope (TEM).

Antibacterial activities of silver and gold nanoparticles:

The antibacterial activities of green synthesized silver and gold nanoparticles were tested against two Gram-negative bacteria like (*Escherichia coli*, *Pseudomonas aeruginosa*) and two Grampositive (*Staphylococcus aureus*, *Bacillus thuringiensis*) bacteria. Preliminary activities were tested using disc diffusion assay method. Approximately 15 ml of molten Mueller-Hinton media was pored in the sterilized Petri discs. The plates were left overnight at room temperature to check for any contamination to appear. The bacterial test organisms were grown in Mueller-Hinton broth for 24

hours. 500 µl broth culture of each bacterial organism (10^6 cfu/mL) were spread on agar using a cotton swab and allow to dry for 30 minutes. The semisolid agars were bored to form wells and wells were filled with colloidal silver and gold nanoparticles solution separately. At the same time reference drugs were placed on the surface of each cultured plates and incubated at $37\pm2^\circ$ C for 24 hours. After 24 hours inhibition zones were measured. Experiments were repeated for four times and the same results were obtained.

Minimum inhibition concentrations (MIC) for tested microorganisms were estimated using standard microdilution method. 10 mL of Mueller-Hinton broth media containing 0, 2, 4, 8....128 μ g/mL sol of silver as well gold nanoparticles were prepared by calculative dilution of the stock colloidal nanoparticles solutions. Four sets of different organisms treated with silver and gold nanoparticles were prepared separately. These were incubated with 10µL of respective bacterial suspension (~10⁶ cfu/mL) at 37±2° C for 24 hours with continuous shaking at 150 rpm. Evaluations of the MICs were carried out by visual inspection of growths in medium containing different concentration of silver and gold nanoparticles. The lowest concentration of silver and gold nanoparticles that inhibited bacterial growth was considered as the MIC for the particular bacterium.

Growth curves for silver nanoparticles exposed and unexposed to *E. Coli* were obtained by adding 4, 8, 16, 32 and 0 μ g/mL Ag nanoparticles separately to the mixture of Mueller-Hinton broth medium and 20 mL culture in five different conical flasks respectively. The cultures were incubated at $37\pm2^{\circ}$ C for 24 hours with continuous shaking at 150 rpm. Growth rates were determined by measuring optical density (OD) at 610 nm through several time series.

RESULTS AND DISCUSSION

. Formation and the stability of silver nanoparticles are confirmed by UV-vis spectral analysis. The UV-vis spectra of silver nanoparticles synthesize from different concentration of silver nitrate are shown in Fig. 1a. The characteristic surface plasmon resonance (SPR) absorption band is observed at 404 nm for yellowish silver nanoparticles synthesized from 0.1mM silver nitrate and soap nut extract. SPR band is shifted to the red with the increase in silver nitrate concentration from 0.1 mM to 100 mM and the corresponding colour changes from yellow - golden yellow –wine red to blackish red. The shifting of SPR peak from 404 nm to 446 nm indicates the increased size of silver nanoparticles and this is also obvious from TEM images (Fig. 2.) Broadening of SPR band for higher concentration of AgNO₃ indicates the heterogeneous size distribution of nanoparticles.



Fig. 1: (a) UV-vis absorption spectra of silver nanoparticles synthesized from Sapindus mukorossi fruit (soap nut) extract and different concentration of AgNO₃ (i) 0.1 mM; (ii) 1.0 mM; (iii) 10 mM; (iv) 100 mM; of AgNO₃ solutions. (b) UV-vis absorption spectra of gold nanoparticles synthesized from (i) 5 mM and (ii) 50 mM HAuCl₄ solution and fruit extract of Sapindus mukorossi.



Fig. 2: TEM micrograph of silver nanoparticles synthesized from (a) 0.1 mM and (b) 100 mM AgNO₃ solutions and soap nut extract.

UV-vis spectra of gold nanoparticles synthesized from 5 mM and 50 mM Chloroauric acid and soap nut extract are shown in Fig. 1b. Figure shows that the intensity as well as full width at half maxima (FWHM) of the SPR band increases with the increase concentration of Au^{+3} ions and absorption peak shifted from 545 nm to 605 nm. Increasing intensity is a measure of increasing concentration of nanoparticles and red shift of SPR band indicate comparable larger size distribution of gold nanoparticles and this observation is well matched with the TEM images shown in (Fig. 3).



Fig. 3: TEM images of gold nanoparticles synthesized from fruit extract of *Sapindus mukorossi* with different HAuCl₄ concentration (a) 5 mM and (b) 50 mM respectively.

The above signature of SPR band in Fig. 1a and 1b reveal the crucial role of soap nut extract both as reducing and stabilizing agent. We also observe very little change in UV-vis (Fig. 1a & 1b) even after two months and it justifies the role of the extract as a good surface passivator for both silver and gold nanoparticles.

The medium becomes acidic and it propagates the reduction automatically. Simultaneously the silver particles are encapsulated by the poly hydroxyl chain of natural surfactants, saponins. The concentrations of surfactants moieties and AgNO₃ determine the size of silver nanoparticles.

Higher resolution transmission electron microscopy (HRTEM) image of particles synthesize from 0.1 mM AgNO₃ solution are presented in Fig. 2a and it shows the morphology and the size of silver nanoparticles. The polydisperse nanoparticles are spherical in size and the average diameters of the particles are in the range 2-5 nm. Whereas larger and uneven shaped particles with diameter 15-25 nm are obtained from 100 mM aqueous AgNO₃ solution (Fig. 2b). The size of the particles at two different AgNO₃ concentrations is in agreement with the observed shift of surface plasmon resonance (SPR) band.

HRTEM images recorded from drop coated films of the gold nanoparticles synthesized from 5 mM HAuCl₄ are shown in Fig. 3a and the diameter of the particles are in the range of 10-20 nm.

Larger size distributions of particle with diameter in the range 20-35 nm are obtained from 50 mM HAuCl₄ solution (Fig. 3b).



Fig. 4: The EDX spectrum of synthesized (a) silver and (b) gold nanoparticles. Strong signals from the atoms in nanoparticles are visible.

The energy dispersive X-ray analysis (EDX) reveals strong signal in the silver region and confirms the formation of silver nanoparticles (Fig. 4a). Metallic silver nanocrystals generally show typical optical absorption peak approximately at 3 keV due to surface plasmon resonance (Magudapatty et al. 2001). Furthermore the sample of gold nanoparticles synthesized from 50 mM Au³⁺ and aqueous fruit extract of *Sapindus mukorossi* is also confirmed by energy dispersive X-ray spectroscopy (EDX) analysis and it is found that the strong signals are from pure gold. (Fig. 4b). Strong signal for Cu in the EDX data for both the sample has come from Cu grid. Other elemental signals are recorded possibly due to elements from components present in fruit extract of *Sapindus mukorossi*.





The antibacterial activities of green synthesized silver and gold nanoparticles are investigated against two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-positive (*Staphylococcus aureus* and *Bacillus thuringiensis*) bacteria. Primarily both silver and gold nanoparticles are able to form inhibition zone against tested bacteria. Fig. 5a shows the extent of inhibition zone formation of silver nanoparticles with references to of the standard antibiotics like Ampicillin (AM), Rifampicin (RR) and Norfloxacin (Nx). The diameters of halo inhibition zones measured in mm scale are presented graphically in Fig. 5b. It has been found that silver nanoparticles exhibit strongest antibacterial activity against *E. Coli* and is almost equivalent to Ampicillin (AM), where as intermediate activities were observed against *S. aureus* and *P. aeruginosa*. Similarly Fig. 6a illustrates the inhibition zones formed by gold nanoparticles with respect to the same antibiotics. The halo inhibition zones (in mm scale) in Fig. 6b suggest that gold nanoparticles have weakest activity against *B. thuringiensis*.





Fig. 6: (a) Extend of inhibition zone formation against four pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus thuringiensis*) of gold nanoparticles with the references to the standard antibiotics like Ampicillin (AM), Rifampicin (RR) and Norfloxacin (Nx). (b) Bar diagram corresponding antibacterial sensitivity against above standard antibiotic.

We also measure the minimum inhibition concentration (MIC) of silver and gold nanoparticles against the above four bacteria. Table-1 shows the MIC values of silver and gold nanoparticles for different bacteria. Silver nanoparticles has highest killing effect on *E. Coli* (32 μ g/mL) followed by *P. aeruginosa* (64 μ g/mL), *S. aureus* (74 μ g/mL) and *B. thuringiensis* (86 μ g/mL). Same trend are observed for gold nanoparticles where lowest MIC is found for *E. Coli* (40 μ g/mL) and highest MIC is obtained for *B. thuringiensis* (98 μ g/mL). Overall results suggest that lower MIC values found for Gram negative bacteria and higher MIC values are found for Gram positive bacteria. In case of Gram negative organisms nanoparticles have to cross the outer membrane along with the thin peptidoglycan layer. Though Gram positive bacteria have no outer membrane but they are surrounded by thicker peptidoglycan layer.⁴⁶ This is the probable reason for higher MIC values for Gram positive bacteria.



Fig. 7: Growth curves of *E. Coli* cells exposed to different concentration of green synthesized silver nanoparticles. The growth curves of *E. Coli* treated with silver nanoparticles are shown in Fig. 7. Different amounts of silver nanoparticles (0, 4, 8, 16 and 32 μg/mL) are treated on *E. Coli* for several hours and optical densities are measured at 610 nm after 6 hours interval. Three phases like lag phase, exponential phase and stationary phase of *E. Coli* growth are clearly observed, where as the decline phases in each growth curve are not clear due to presence of live bacteria with the dead one during

the measurements of optical densities.

	Gram Positive Bacteria	MIC (μg/mL) Ag-nanoparticles	MIC (μg/mL) Au-nanoparticles
1	Staphylococcus aureus (ATCC-25923)	74	86
2	Bacillus thuringiensis (ATCC-12228)	86	98
Gram Negative Bacteria			
3	Escherichia coli (ATCC 25922)	32	40
4	Pseudomonas aeruginosa (ATCC 27853)	64	68

 Table 1: Minimum inhibitory concentrations (MICs) of green synthesized silver and gold nanoparticles for four individual strains.

Untreated *E. Coli* reach exponential phase rapidly, but when exposed to 4, 8 and 16 μ g/mL silver nanoparticles, the tested bacteria cells are lagged to 12, 24 and 36 hours respectively. With the increasing dose of silver nanoparticles the delayed are more evident. When the amount of AgNPs is 32 μ g/mL, no growth of *E. Coli* are observed till upto seven days, and it indicates that the minimum inhibitory concentration (MIC) of silver nanoparticles to *E. Coli* is 32 μ g/mL.

CONCLUSION

In this study we demonstrated a novel green synthesis of silver and gold nanoparticles using *Sapindus mukorossi* fruit extract. The soap nut is environment-friendly bio-surfactant. Here the bio-extract act both as reducing as well as stabilizing agent for the synthesis of nanoparticles. The

spectroscopic characterization using UV-vis, TEM and EDX support the formation and stability of the green synthesized silver and gold nanoparticles. UV-vis spectral analysis shows that the size of nanoparticles can be regulated by changing the concentration of AgNO₃ and HAuCl₄. The antibacterial activity of silver and gold nanoparticles were analyzed and it was found that silver nanoparticles showed higher activity compare to gold nanoparticles which is nearly equivalent to the known antibiotic Ampicillin. Minimum inhibitory concentration (MIC) of silver nanoparticles for *E*. *Coli* is 32 µg/mL where as gold nanoparticles has MIC value 40 µg/mL. These eco-friendly Ag and Au nanoparticles are compatible for pharmaceutical and bio-medical application and also for large scale industrial production.

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