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IN VITRO EVALUATION OF ANTIBIOTIC CONJUGATED BIOGENIC GOLD NANOPARTICLES BY *NEOSARTORYA UDAGAWAE*

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ABSTRACT

Gold nanoparticles (AuNPs) were synthesized in a novel single step method by fungus *Neosartorya udagawae* and characterized by UV-Vis spectrophotometry, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Gold nanoparticles have the absorption maxima at 536 nm and size ranges from 50 to 53nm. Gold nanoparticles were conjugated with antibiotics Amoxicillin, Streptomycin and Gentamycin. Conjugation was confirmed by UV–Vis, SEM and TEM. Conjugation process enhanced the antimicrobial efficacy of the antibiotics over free AuNPs and standard drugs against *E. coli*. The antibacterial effect was confirmed by antibody diffusion method and AuNPs conjugated with antibiotic Streptomycin are highly effective against *E. coli* than Amoxicillin and Gentamycin conjugates due to greater stability and higher anti-microbial activity.

KEYWORDS: **Gold nanoparticles (AuNPs), *Neosartorya udagawae, E. coli,* conjugation, SEM, TEM.**

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INTRODUCTION

Nanotechnology has recently become one of the most active areas of research in modern material science with broad potential applications in fighting major health problems of the world[1] by producing more effective drugs with functionalized nanoparticles. Various protocols are in practice to synthesize nanoparticles in physical and chemical methods by chemical reduction of salts, laser ablation, ultrasonic fields, aerosol techniques, UV irradiation and reducing Gold photo chemically and lithography. But their inherent flaws include impurity, use of toxic chemicals as reducers and hazardous by-products makes them incompatible for therapeutic applications in biological systems[2]. These shortcomings insisted to explore novel green techniques for synthesizing high-yielding, low cost, non-toxic, environmental friendly and biocompatible nanoparticles by using microorganism, enzymes and plant extracts[3]. Among all Gold nanoparticles are most favored for their functional ability[4], non-toxicity[5], high stability, simple fabrication of optimal size and shape. Moreover Colloidal gold solutions have cytotoxic properties used in pharmacology, medicine and biocompatible for biological applications that include biosensors, DNA labeling, vapor sensing, photothermal therapy, tissue/tumor imaging, drug-delivery, food, water purification industry[6] and coating to wide variety of surfaces. Stability of the nanoparticles plays vital role in biomedical applications and stability is directly connected to the reducers used for synthesis, like tri-sodium citrate reduces highly stabile narrow sized silver nanoparticles[7]. Microorganism cause infectious diseases in humans, animals and plants. Conjugation process enhances the efficiency of antibiotics against microorganism whereas vancomycin conjugated AuNPs show more antimicrobial activity against vancomycin resistant enterococci (VRE)[8] and Cefaclor (a second-generation β-lactam antibiotic) reduced AuNPs have potent antimicrobial activity on both Gram positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) compared to Cefaclor and AuNPs alone[9]. AuNPs conjugated streptomycin and kanamycin showed greater stability and higher activity than its free form in *E.coli* DH5α[10]. AuNPs when used as conjugates with antibiotics generate holes in the cell wall of bacteria causing the leakage of cell contents and cell death, further AuNPs bind with DNA of bacteria and inhibit the uncoiling and transcription[11]. Many techniques are being followed to functionalize AuNPs with amino acids, glutathione and polyethylene glycol etc[12]. The main objective of this study is to find novel and simple technique to synthesise gold nanoparticles and the conjugation process of antibiotics with AuNPs and also to investigate *in vitro* antimicrobial activity of AuNP-conjugates. The antibiotics Amoxicillin, Streptomycin and Gentamycin were separately conjugated with AuNPs. Stability, characteristics and antimicrobial activity of the conjugates were investigated keeping them at room temperature for a long time.

MATERIALS AND METHODS

Chemicals

Mueller Hinton agar media, Antibiotics – Amoxicillin, Streptomycin, Gentamycin and Chloroauric acid ($HAuCl_4 \cdot 3H_2O$) were purchased from Hi media.

Synthesis of biogenic Gold nanoparticles

Seven days old fungal mat was used for the synthesis of AuNPs. Fungal mat was washed thrice with distilled water to remove media components and incubated in 6 mM gold solution at 28 ±1^{o}C, an immediate change of the color was observed. After 24h the ruby red color mixture was centrifuged at 7000 RPM for 20 min to separate colloidal particles from the mixture. The supernatant solution was collected and dried in a vacuum dryer to obtain dried powder of Goldnanoparticles[13].

Characterization of Goldnanoparticles

Bio reduced gold nanoparticles were monitored by UV–Vis spectra after diluting the sample with deionized water[14]. A Perkin-Elmer Lamda-45 spectrophotometer was used to determine the absorption maxima of synthesized AuNPs for preliminary size characterization between 200 nm to 800 nm at a resolution of 1 nm using 1cm path quartz cuvette by Spec rod 210 plus - 223F1427. Formation of nanoparticles, size distribution and concentration were studied by TEM. The TEM samples were prepared by placing a drop of the aliquots on carbon coated copper grids of aqueous suspension of AuNPs and allowed the water to evaporate[15]. Size distributions of the resulting nanoparticles were estimated using the micrographs of AuNPs using Technai Sprit HT: 120KV Electron Source: LaB6. SEM analysis was carried to confirm the results of TEM. Micrographs were obtained using a JEOL (6360) JED-2300 analysis station operating at 200 KV. (Model: Qunata 250 Detector: Everhart Thornley Detector Electron Source: Tungsten).

Synthesis of AuNP-antibiotic conjugates

A stock solution (10 mg/ml) of Amoxicillin, Streptomycin and Gentamycin were prepared and stored at −20°C. For the conjugation process, 0.5 ml antibiotic stocks were added drop wise to 5 ml of washed AuNPs solution under stirring for 18 h condition and these AuNP-antibiotic conjugates were stored at 4°C and used for further experiments[16].

Characterization of AuNP-antibiotic conjugates

The UV-Visible absorption spectra of the AuNP-antibiotic conjugates were investigated. All the three AuNP-antibiotic conjugates and free AuNPs were prepared by drying on a carbon coated copper grid and observed under TEM with an accelerating potential of 200 KV. SEM analysis carried out to study the structure and morphology of the conjugates. AuNP conjugated antibiotics and free AuNPs were lyophilized on glass slides and samples were then observed under SEM (FEI, Czech Republic)

Antimicrobial activity

A clinical isolate of *E.coli* was used for antibiotic assay. Antibacterial activity of AuNPs was studied by agar-well-diffusion method[17]. *E. coli* suspension was added to the sterile Mueller Hinton agar at 45^0 C and the mixture was solidified on a Petri dish. A 20 ml volume of the medium was poured into a Petri dish (diameter, 90 mm) on a horizontally leveled surface. After the medium had solidified, 6 mm diameter wells were made in the agars (four wells per dish) that were equidistant from one another and from the dish edge. The wells received 30µl of sterile distilled water for control; equal volumes of the free antibiotic solution, free AuNPs and AuNP antibody conjugates. The Petri dishes were incubated in a thermostat at 37^0C for 24 h. After incubation the diameter of the zone of bacterial growth inhibition was measured with an accuracy of ± 0.1 mm and mean inhibition zone diameters were determined. All experiments were repeated thrice.

RESULTS AND DISCUSSION

Gold nanoparticles formed instantly within a few seconds by the fungal mat *Neosartorya udagawae* when incubated in 6mM concentration of Tetra chloroauric (III) acid. AuNPs formation was observed visually by change of color to ruby pink.

Characterization of Goldnanoparticles

The SPR absorption maxima of AuNPs found at 536 nm in UV-Visible spectroscopy (Figure.1) which indicates the nano status and stability of the nanoparticles. The UV-Vis absorption maximum is analogous to the Gold nanoplates synthesized by *Aspergillus niger*[18]. SEM analyses clearly show the gold nanoparticles in spherical shape and the size ranges from 50-53 nm (Calculated from SEM and TEM experiments). SEM images were analogous to the results of AuNPs from *Arthobacter Sp. 61B* [19]. The TEM micrograph (Figure.2) show the formation of nano triangles, sphericals and size of the sphere is smaller than the triangles. The high resolution TEM image shows the well-defined lattice fringes indicating both types of particles are highly crystalline in nature[20]. The gold nanoparticle size is analogous to the results of gold nanoparticles from *penicillin rugulosum*[21].

Characterization of conjugated Goldnanoparticles

AuNPs antibiotic conjugates were noted by UV–visible spectroscopy. The graphs (Graph1,2 and 3) represent the spectra of AuNPs conjugated with Amoxicillin, Streptomycin and Gentamycin respectively. However SEM images shown distinct structures, the absorption maxima at 538nm, 540nm and 545nm clearly indicating the stability of AuNPs after conjugation with antibiotics by producing strong peak in between 500 – 600 nm.

Transmission Electron Microscopy

The surface morphology of TEM (Figure.3) shows that the AuNPs adhere to surface in a scaly pattern and smaller sized particles were almost spherical in shape. The antibiotic conjugates were polydisperse and irregular shaped particles varied from spherical, triangle and truncated triangles with a thin shell of antibiotics creating a tube like structures with different dimension confirms the conjugation process.

Scanning Electron Microscopy

The micrographs of SEM (Figure 4) show the structure and morphology of free AuNPs before and after conjugation. Three antibiotic conjugates display distinct structures due to the different absorption of antibiotics on the nanoparticle surface and seem relatively stable. The enhanced stability of the antibiotic conjugates attributed to the close association of antibiotics with AuNPs which in turn increase the bond energy of antibiotics.

Figure 1
The UV-Visible spectroscopy of AuNPs

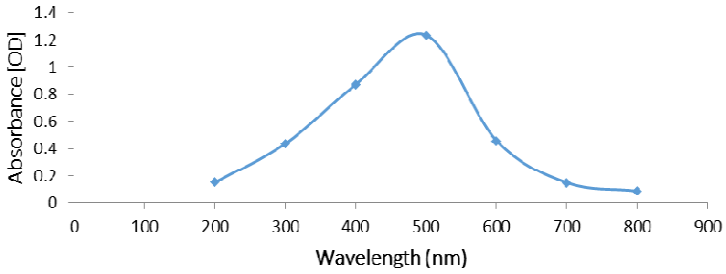
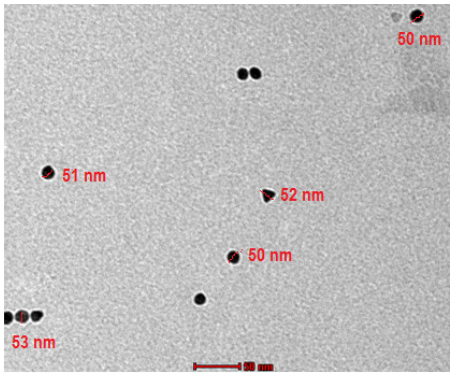
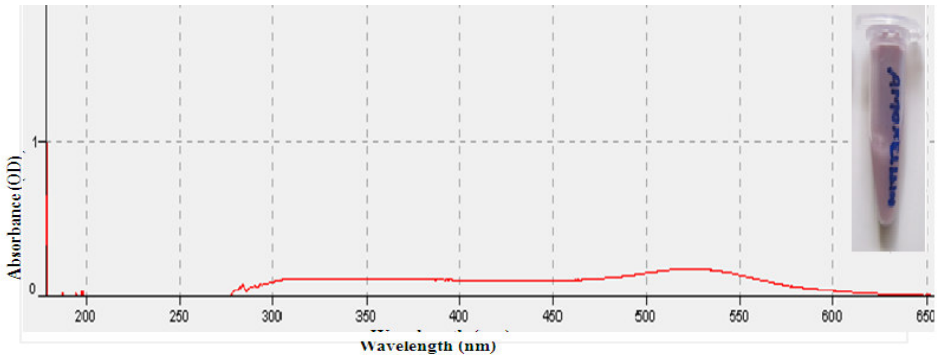


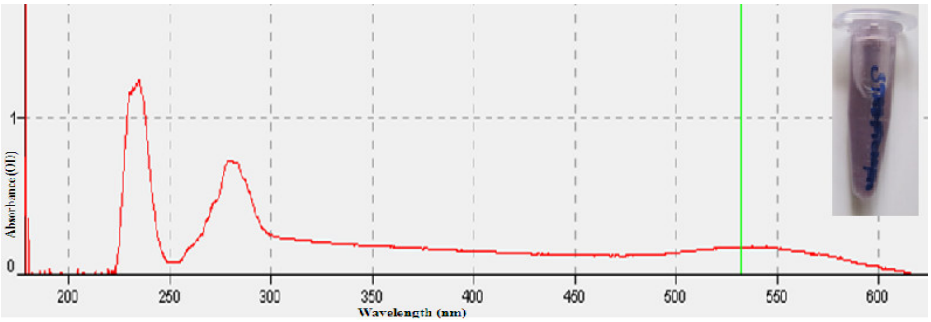
Figure 2
The TEM micrograph AuNPs



Graph 1
UV – Vis absorption spectra of AuNPs conjugated with Amoxicillin



Graph 2
UV – Vis absorption spectra of AuNPs conjugated with Streptomycin



Graph 3
UV – Vis absorption spectra of AuNPs conjugated with Gentamycin

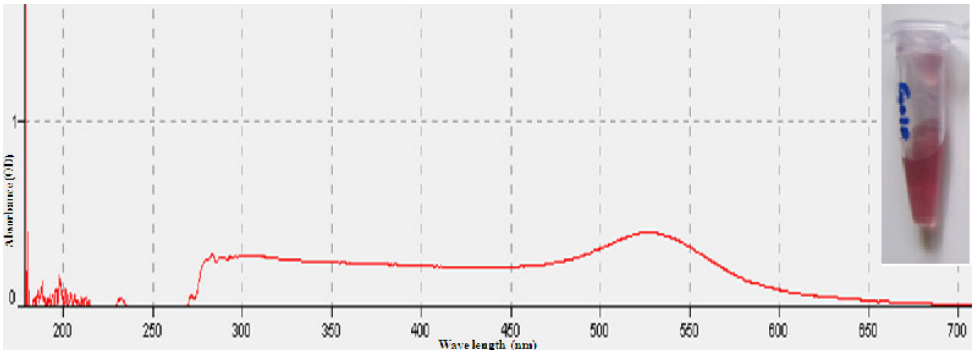


Figure 3
TEM of a) Free AuNPs b) Amoxicillin conjugated AuNPs c) Streptomycin conjugated AuNPs d) Gentamycin conjugated AuNPs

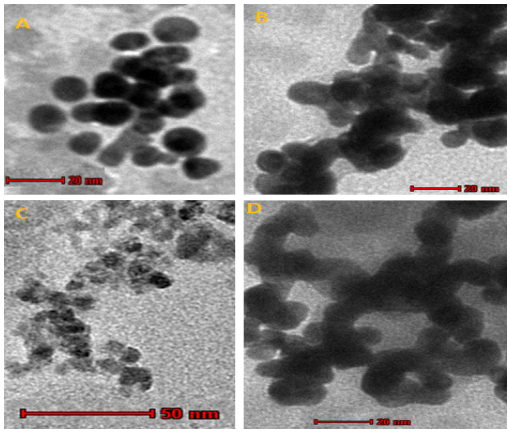


Figure 4
SEM of free and Antibiotic conjugated AuNPs. a) Free AuNPs b) Amoxicillin conjugated AuNPs c) Streptomycin conjugated AuNPs d) Gentamycin conjugated AuNPs.

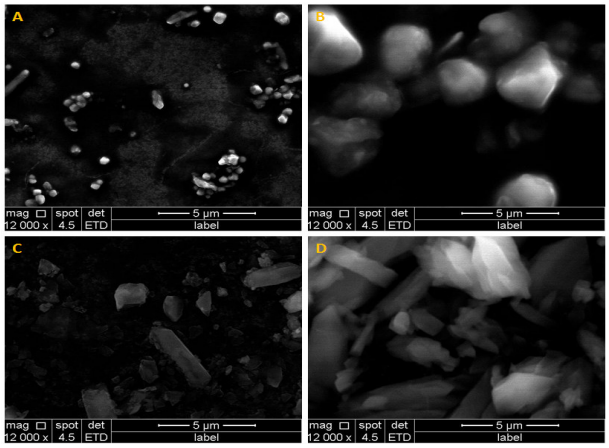
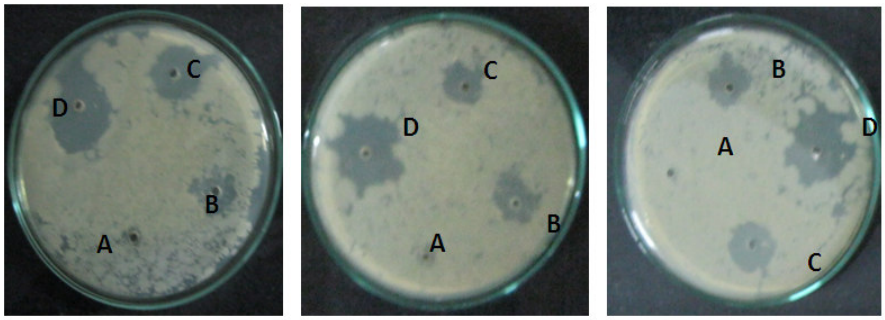


Table 1
The Zone of inhibition of free AuNPs, standard drugs and Antibiotic conjugated AuNPs

Organism	*E.coli*
Control (sterile distilled water)	NIL
Free AuNPs	5 mm
Free Amoxicillin	11 mm
AuNP conjugated Amoxicillin	14 mm
Free Streptomycin	18 mm
AuNP conjugated Streptomycin	24 mm
Free Gentamycin	14 mm
AuNP conjugated Gentamycin	20 mm

Figure 5
Assessment of antibacterial activity of antibiotics conjugated AuNPs along with respective free antibiotics in E. coli by agar well diffusion method



A. Control
B. AuNPs
C. Amoxicillin
D. AuNPs conjugated Amoxicillin

A. Control
B. AuNPs
C. Streptomycin
D. AuNPs conjugated Streptomycin

A. Control
B. AuNPs
C. Gentamycin
D. AuNPs conjugated Gentamycin

Antibacterial assay

The antimicrobial activity of antibiotics was analyzed by the zone of inhibition around the microbial colonies against *E.coli* which is a common cause of gastroenteritis. Enterotoxigenic *E. coli* (ETEC) alone is responsible for 280 to 400 million of diarrhoea cases leading to malnutrition and 380,000 deaths annually among the different strains of *E.coli*[22] which has developed antibiotic resistance. Conjugation of nanoparticles enhanced antimicrobial activity and stability of nanoparticles due to the presence of strong reducing agents and delocalization of the electrons in the carbonyl group in β-lactam rings of antibiotics. Conjugation by UV increases the bond energy between antibiotics and further cross link the antibiotics with nanoparticles, which in turn increase the stability as well as antibiotic activity[9] and also enhance the concentration of antibiotics inside the cells. The mechanism of nanoparticles is to attach the cell membrane and then to penetrate inside the bacteria where membrane contains sulfur-containing proteins and nanoparticles interact with these proteins in the cell as well as with the phosphorus-containing compounds like DNA and preferably attack the respiratory chain, cell division and finally leading to cell death[23]. Thus, in all the cases, the stability and antimicrobial activity of AuNP conjugated antibiotics increased significantly by conjugation process. The zone of inhibition (Figure 5) of antibiotic conjugates was higher than the free AuNPs and standard drugs (Table 1). Hence the conjugation process increases the efficacy and AuNPs conjugated with Streptomycin is more effective against pathogen *E. coli.*

CONCLUSION

In conclusion, our study demonstrates a novel single step biogenic synthesis of AuNPs by fungus *Neosartorya udagawae.* The SPR absorption maxima at 536 nm in UV confirm the formation of AuNPs. Gold nanoparticles synthesized were spherical and triangle in shape and size ranges from 50-53 nm. AuNP- antibiotic conjugates of Streptomycin, Amoxicillin and Gentamycin were highly stable in UV, absorption maxima were observed between 500-600nm. The conjugation process increased the stability and antimicrobial activity of antibiotics against drug resistant pathogen *E. coli.* AuNPs conjugated with Streptomycin are more effective than free AuNPs, Amoxicillin and Gentamycin. The process involved is simple, cost-effective, eco-friendly and can be effectively used against multi-drug resistant organisms in biomedical applications.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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