International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (3): 388-394
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Biosynthesis, Characterization and Evaluation of Antimicrobial Activity of Copper and Silver Nanoparticles Synthesized using *Ricinus communis*

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Received: 23 Mar 2019 / Accepted: 25 Apr 2019 / Published online: 1 Jul 2019
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Abstract

The antimicrobial activities of copper nanoparticles (CuNPs) and silver nanoparticles (AgNPs) are well known and research is currently underway to develop protocols using nanoparticles for treatment of diseases caused by an increasingly growing number of antibiotic resistant microbes. In the present work, we have synthesized copper and silver nanoparticles from various tissues of the plant *Ricinus communis* (Castor bean). The effect of change in four different environmental variables on the synthesis of nanoparticles was investigated viz. concentration, pH, temperature and incubation period. Successful biosynthesis of nanoparticles was indicated by a change in the colour of the solution. Characterization of the synthesized nanoparticles was done using UV-Visible spectroscopy and SEM (Scanning Electron Microscopy). The antimicrobial and synergistic effects of the synthesized nanoparticles were studied on antibiotic resistant strains of *E. coli* and *S. aureus*. CuNPs did not exhibit any antimicrobial activities whereas AgNPs did exhibit antimicrobial activity. No synergistic effect was observed between CuNPs and AgNPs.

Keywords

Antimicrobial activity, copper nanoparticles, *Ricinus communis*, silver nanoparticles.

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INTRODUCTION

The term "nanoparticles" is used to describe particles with size in the range of 1–100 nm, in at least one of the three dimensions. In this size range, the physical, chemical and biological properties of the nanoparticles change in fundamental ways from the properties of both individual atoms/molecules and of the corresponding bulk materials. Engineered nanomaterials are generally produced by the "Top-down" approach or "Bottom-up" approach [1]. In the Top-down approach, large, bulk matter is reduced into nanoscale structures by chemical, mechanical or

physical methods. In the Bottom-up approach, atomic or molecular components are assembled through chemical reactions or physical processes to produce nanoscale structures [2].

Use of plants in synthesis of nanoparticles is novel leading to truly green chemistry. Plants and plant extracts have been explored under different environmental conditions to successfully produce metallic nanostructures. Plant extract is the most commonly used form of plant tissue to produce metallic nanoparticles. The synthesis of nanoparticles using plant extract eliminates the need for elaborate processing of cell growth and culture preparation when using microorganisms for synthesis of nanoparticles. The first report of synthesis of silver and gold nanoparticles involved use of Lucerne (*Medicago sativa*) leaves. However, the protocols for preparing nanoparticles using plant extracts vary. Distinctive metallic nanoparticles can be synthesized under diverse environmental conditions [3].

Copper (Cu) is a microelement required for the development of plant. It exists as Cu^{2+} and Cu^{+} ions under physiological condition. Concentration required for normal development of plant is from 10^{-14} to 10^{-16} M, below which deficiency occurs. However higher than optimum concentrations show toxic effects [4].

Copper nanoparticles (CuNPs) are of great interest due to their excellent physical and chemical properties and low cost of preparation. CuNPs have wide applications as heat transfer systems, antimicrobial materials, super strong materials, sensors and catalysts. CuNPs are very reactive because of their high surface-to-volume ratio and easily interact with other particles thus increasing their antimicrobial efficiency. Colloidal copper has been used as an antimicrobial agent for decades. Copper nanoparticles have a strong antibacterial activity and are able to decrease the bacterial concentration by 99.9% [5].

The antimicrobial activity of CuNPs is linked with ions that are released from nanoparticles. The activity is further enhanced by its small size and high surface area-to-volume ratio that allows them to interact closely with microbial membranes. Antimicrobial activity is due to its tendency to alternate between its cuprous - Cu (I) and cupric - Cu (II) oxidation states, differentiating it from other trace metals. The redox cycling between the two oxidation states results in the production of hydroxyl radicals that subsequently bind with DNA molecules and lead to disorder in the helical structure due to cross-linking within and between the nucleic acid strands and damage to essential proteins by binding to the sulfhydryl amino and carboxyl groups of amino acids. This denatures proteins and makes enzymes ineffective. It inactivates cell surface proteins necessary for transport of materials across cell membranes, thus affecting membrane integrity and membrane lipids. Copper ions inside bacterial cells also disrupt biochemical processes. However, the exact mechanism behind the toxicity is not known. Thus, the denaturing effects of Cu ions on proteins and enzymes in microorganisms gives Cu its antimicrobial characteristics [4].

Due to its close association with human history, silver has been used in several applications including dentistry in the form of amalgams with mercury for fillings to repair tooth decay. It has also been used since ancient times for its microbicidal properties. Silver salt and its colloidal formulations have been used to treat ulcers, burns and chronic wounds, sepsis, acute epididymitis, tonsillitis, and infections and to prevent eye diseases in infants; but its use was discontinued due to the interfering effects of the salts and the development of new and effective antibiotics [6]. However, since the last decade, nano silver made a remarkable comeback owing to its high surface area-to-volume ratio and size-dependent unique optical, electrical, and thermal properties. Silver nanoparticles are now one of the most commercialized nanomaterials having applications in over 200 products such as antimicrobial coatings, medical devices, molecular diagnostics and photonic devices, sensors, textiles, home water purifiers, cosmetics, electronics, household appliances, conductive inks, pastes, and fillers.

Unlike commercial antibiotics, AgNPs do not exhibit their effects in a single specific way. Combinations of mechanisms such as disruption of cellular morphology, inactivation of vital cellular enzymes and proteins, DNA condensation, loss of DNA replication, depletion of ATP, protein denaturation, inhibition of ribosome interaction, accumulation at lethal concentration in cell, generation of reactive oxygen species (ROS), oxidative stress, and modulation of cellular signalling make AgNPs ideal for targeting a broad range of microorganisms. AgNPs show antimicrobial activities against various infectious organisms, e.g., *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Vibrio cholera* [6].

Phytochemicals are biologically active chemical substances synthesized by plants. Some are primary metabolites while the rest are secondary metabolites and have different biological properties. The different phytochemicals produced by plants include

alkaloids, steroids, tannins, etc. In the plants, these phytochemicals play a major role in the defence mechanism against plant damage caused due to various environmental hazards such as drought, pollution, stress, exposure to UV radiation and against diseases and infections caused by various pathogenic organisms. Also, they contribute to the aroma, colour and flavour of the plants. These phytochemicals accumulate in different parts of the plants. Their levels vary in different plants depending on various factors and conditions. Phytochemical screening of the extracts (leaves, stem, flower, roots and seeds) to check for the presence of bioactive compounds is usually carried out as correlation is sometimes observed between presence of a certain secondary metabolite and nanoparticle synthesis [7, 8].

Ricinus communis Linn. (Family – *Euphorbiaceae*; common name – castor bean) is an evergreen soft-woody shrub or small tree (around 1.5 m) which grows annually. It has another common name - palm of Christ or Palma Christi - that derives from the ability of castor oil to heal wounds and cure ailments. Castor oil has many uses in medicine and other fields. Ethanolic extract of *Ricinus communis* root bark has antihistamine and anti-inflammatory properties [9]. Methanolic extract of *R. communis* roots have significant anti-inflammatory activity in acute and chronic inflammatory models in rats. The leaf extracts, too, have antimicrobial activities [10, 11].

The overall aim of this study was to prepare and phytochemically analyse aqueous extracts prepared by using various tissues of the plant *Ricinus communis* and to then use these extracts to synthesize copper and silver nanoparticles by treating solutions of copper sulphate and silver nitrate with the extracts, to characterize the synthesized nanoparticles using UV-Visible spectroscopy and Scanning Electron Microscopy and to investigate the antimicrobial and synergistic effects of the synthesized nanoparticles on antibiotic resistant strains of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

MATERIALS AND METHODS

Plant material collection:

Whole *Ricinus communis* plants were collected from along the edge of the arterial road near the campus of old VIVA College, Virar (West), District – Palghar in Maharashtra State, India.

The different tissues (leaves, stems, branches, flowers, seeds) were thoroughly washed, first under normal tap, and later using distilled water. The tissues were allowed to dry.

Preparation of extract:

The extracts using the different tissues of *Ricinus communis* were prepared by weighing 6 g of dry tissue and crushing it with 20 ml of distilled water using a mortar and pestle. The contents were then transferred to clean 100 ml Erlenmeyer flasks by filtering through Whatman No.1 filter paper. The total volume in each flask was then made 100 ml using distilled water.

Synthesis of copper and silver nanoparticles:

3 ml of the various extracts prepared were added to 5 ml each of 1 mM, 5 mM and 10 mM of $CuSO_4$ and $AgNO_3$ solutions respectively. The biosynthesis of both nanoparticles was investigated using different environmental parameters.

The different parameters investigated (including concentration, mentioned above) were incubation period (24, 48 and 72 hours), pH (5, 7 and 9) and incubation temperature (4, 27 and 37°C).

Reduction of copper and silver ions into copper and silver nanoparticles on reaction with the plant extracts was indicated by colour change. Copper nanoparticles exhibited greenish-blue to bluish-green colour in aqueous solution while the silver nanoparticles exhibited light to dark brown colour in aqueous solution.

Phytochemical analysis:

The presence of reducing sugars was tested using the method described by Fehling [12]. Monosaccharides were tested using the Barfoed's test [13]. The presence of pentose sugars as detected by the Bial's orcinol test [14] whereas hexose sugars were detected by the Seliwanoff test [15]. The presence of alkaloids was detected using the Dragendorff's reagent [16] while the tannins were detected by precipitating them with a solution of lead acetate. Finally, steroids were detected by the Salkowski reaction [17].

Antibacterial activity:

For this study, cultures antibiotic resistant strains of test bacteria *E. coli* and *S. aureus* were procured from Biotechnology Department of VIVA College.

The antimicrobial (antibacterial) activities of each of the plant-mediated nanoparticles were assessed and also their combined activity/effect (synergistic activity/effect) was assessed on the test organisms. Saline suspensions of the above cultures were prepared by inoculating the cultures. Sterile Mueller Hinton (MH) agar plates were prepared for the culturing of the test organisms.

100 µl of overnight grown cultures of both the test organisms were spread plate on St. MH agar plates using a sterile glass spreader. Using sterile forceps, a sterile Whatman No.1 filter paper strip (1 cm x 7.5

cm) was dipped in the solution containing AgNPs (obtained from flower and leaf tissue extracts with 10 mM $AgNO_3$, incubated at RT for 72 hours) and placed in the centre of separate MH agar plates seeded with *E. coli* and *S. aureus*. Similarly, the antibacterial activity of CuNPs was tested using the solution containing CuNPs (obtained from flower and leaf tissue extracts with 10 mM $CuSO_4$, incubated at RT for 72 hours) and placed in the centre of separate MH agar plates seeded with *E. coli* and *S. aureus*.

To determine the synergistic effect of CuNPs and AgNPs, a sterile Whatman No. 1 filter paper strip (1 cm x 7.5 cm) was dipped in the solution containing AgNPs obtained using the flower tissue extract and placed in the centre of the MH agar plate of *E. coli* and *S. aureus*. A second sterile filter paper strip was then dipped in the solution containing CuNPs obtained using the flower extract and placed at a right angle on top of the previous strip.

The same procedure was repeated for solutions containing AgNPs and CuNPs obtained from leaf tissue extract.

All the plates were then incubated at 37 °C for 24 hours. The antimicrobial activity was evaluated in terms of inhibition around the edge of the filter paper strips containing the nanoparticles. Synergistic effect was evaluated in terms of an increase in the apparent size of the zone of inhibition at the junction of the strips as compared to the inhibition around the individual strips.

RESULTS AND DISCUSSION

Synthesis of nanoparticles:

Reduction of the metal ions to metal nanoparticles on being exposed to various plant extracts was followed by colour change. Varied intensities of reaction mixture corresponding to the production of nanoparticles were observed, with the colour intensifying with an increase in the concentration of salt solution and an increase in incubation temperature. Leaf and flower extracts showed promising results with very less effect of pH on the colour change. Hence, the amount of nanoparticles synthesis can be controlled by varying the concentration of salt or by increasing the time / temperature of incubation. All of the three criteria are easy to control. The absence of any effect on the synthesis of nanoparticles is an added advantage since the synthesis can be carried out at a neutral pH without any loss in the yield of nanoparticles.

Other tissue extracts did not show any significant change in the colour of the solution after incubation indicating absence of nanoparticle synthesis by these tissues.

Characterization by SEM analysis and UV-Vis spectrophotometer:

SEM analysis was carried out on the synthesized copper nanoparticles (Flower extract with 10mM $CuSO_4$, 72 hours) (Figure 1). The size of the nanoparticles was greater than 250 nm. The greater size maybe due to the presence of other plant secondary metabolites or proteins surrounding the nanoparticles or maybe due to agglomeration.

SEM analysis was carried out on the synthesized silver nanoparticles (Leaf extract with 10mM $AgNO_3$, 72 hours) (Figure 2). The size of the nanoparticles was less than 100 nm. The average nanoparticle size ranged from 62-72 nm.

The prepared aqueous solutions of CuNPs showed an absorption band between 250 – 320 nm for salt solutions incubated with leaf (Figure 3) and flower (Figure 4) extract, which is a typical absorption band of spherical CuNPs due to their surface plasmon.

The prepared aqueous solutions of AgNPs showed an absorption band between 250 – 350 nm for salt solutions incubated with leaf (Figure 5) and flower (Figure 6) extract, which is a typical absorption band of spherical AgNPs due to their surface plasmon.

Phytochemical analysis:

The presence of alkaloids was detected in the leaf as well as the flower extracts. Reducing sugars were detected only in the leaf extract whereas steroids and tannins were detected in both the extracts.

Antimicrobial analysis and evaluation of synergistic action:

Antimicrobial assay of the biosynthesized copper nanoparticles against both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) microorganisms revealed that the nanoparticles were not capable of inhibiting growth of both the cultures. However, the biosynthesized silver nanoparticles showed antimicrobial activity against both the microorganisms (Figures 7, 8, 9, 10). However, the inhibition only in the immediate vicinity of the filter paper strips dipped in the AgNP solution suggests that the nanoparticles would probably be more suited in applications in which the nanoparticles come in direct contact with the microbes such as topical creams, deodorants, etc.

Synergistic action of both nanoparticles was assessed since copper failed to inhibit the growth of organisms, and no synergistic relation between the two was observed.

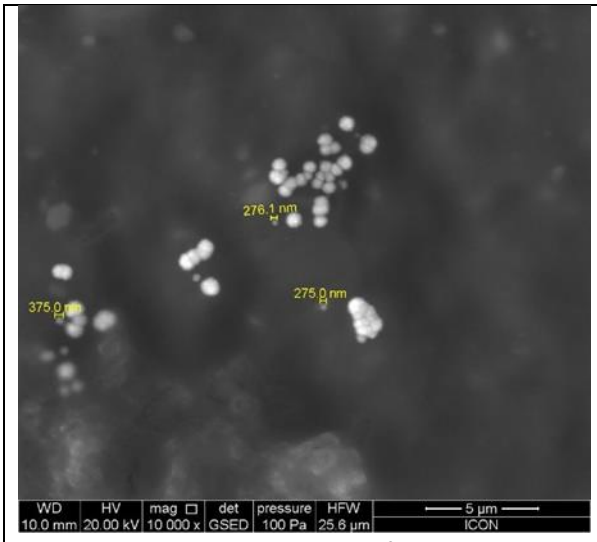

Figure 1: SEM image of CuNPs

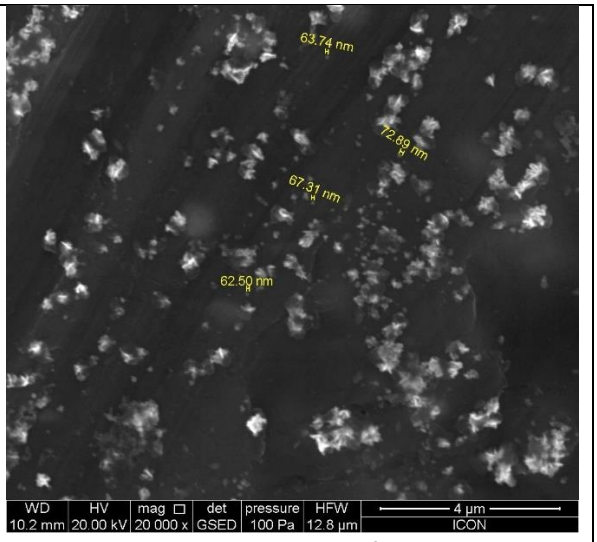

Figure2: SEM image of AgNPs

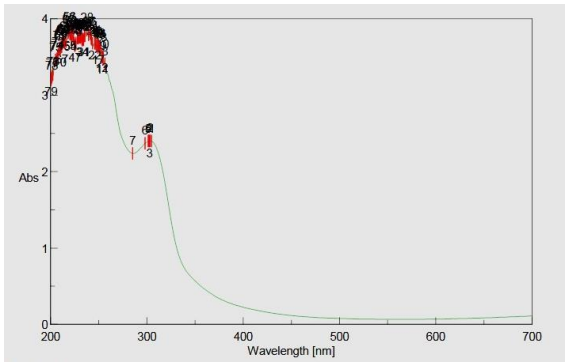

Figure 3: UV-Vis spectra of CuNPs from leaf extract

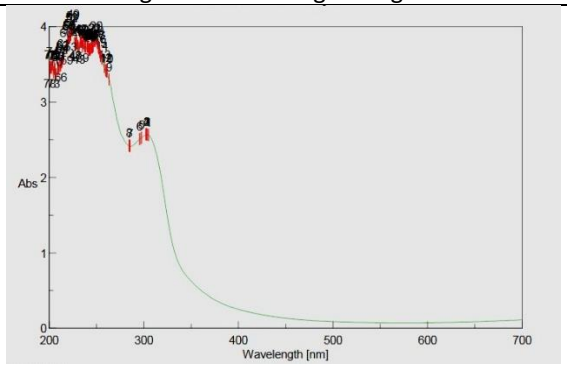

Figure 4: UV-Vis spectra of CuNPs from flower extract

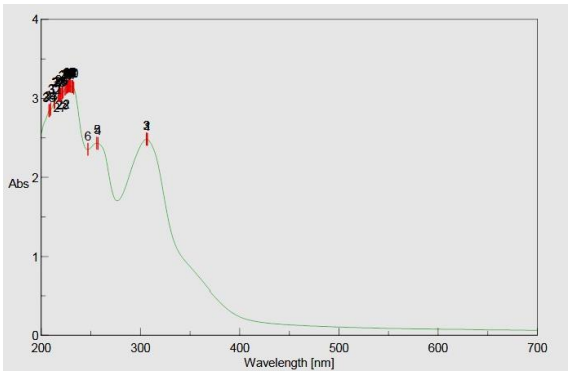

Figure 5: UV-Vis spectra of AgNPs from leaf extract

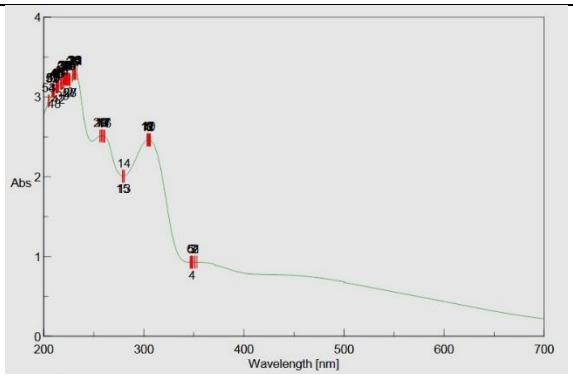

Figure 6: UV-Vis spectra of AgNPs from flower extract

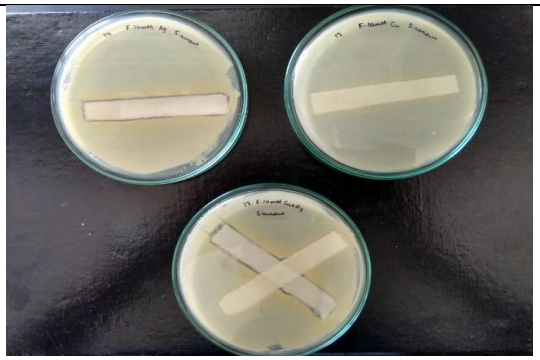

Figure 7: Antimicrobial activity of AgNPs and CuNPs synthesized from aqueous flower extract on *S. aureus*

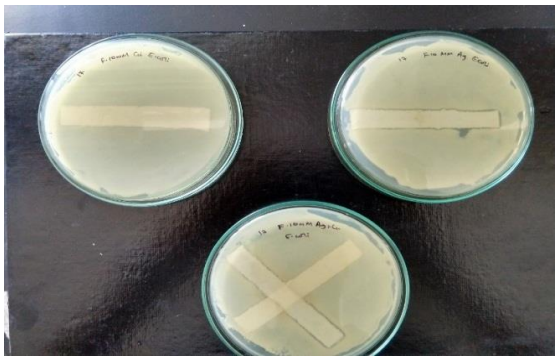

Figure 8: Antimicrobial activity of AgNPs and CuNPs synthesized from aqueous leaf extract on *S. aureus*

Figure 9: Antimicrobial activity of AgNPs and CuNPs synthesized from aqueous leaf extract on *E. coli*

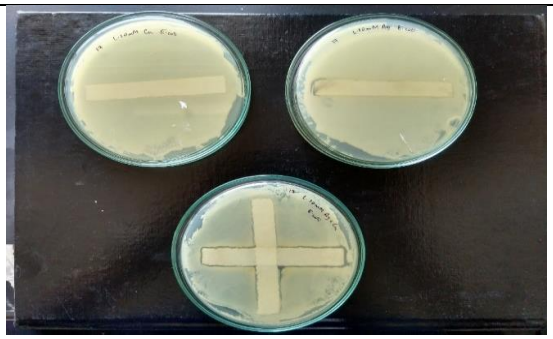

Figure 10: Antimicrobial activity of AgNPs and CuNPs synthesized from aqueous Flower extract on S. aureus

CONCLUSION

The rapid biological (green) synthesis of Copper and Silver nanoparticles using the flower and leaf extract of *Ricinus communis* plant provides an environmentally friendly, simple and efficient route for the synthesis of nanoparticles as compared to the expensive physical and chemical methods applied in industries for commercial production of nanoparticles. Phytochemical analysis of *R. communis* leaf and flower extract was carried out and the presence of alkaloids, tannins and steroids was detected. Successful bio reduction of copper and silver ions by plant metabolites in the extracts into nanoparticles was confirmed. The amount of nanoparticles synthesized were affected by the concentration of salt, temperature, incubation time and type of tissue used in its preparation, but not by the pH of the experimental setup. Characterization of the by SEM and UV-Vis spectroscopy revealed presence of large agglomerates for CuNPs but nano-sized particles for AgNPs. Antimicrobial activity was exhibited by AgNPs but not by CuNPs. No synergistic effect was observed when the two nanoparticles were used together.

The flower and leaves of the plant showed a promise in its use for synthesis of CuNPs and AgNPs. While the CuNPs were large in size, the AgNPs were on the nanoscale and showed characteristic absorption spectra. Their activity against pathogenic bacteria may find application in various medical and cosmetic industries.

This simple procedure for synthesis has advantages such as cost-effectiveness, compatibility for medical and pharmaceutical applications as well as large scale production.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Vihang Ghalsasi for the translation of original classic research papers from German to English.

AUTHOR CONTRIBUTIONS

Umar Khan carried out the laboratory work, Mohammed Mubeen Shaikh co-supervised the laboratory work, Vikas Gupta contributed to the writing, Ganesh Lad conceived and coordinated the study and wrote the manuscript. All authors gave final approval for publication.

CONFLICT OF INTEREST

The authors do not have any competing or conflict of interests.

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